MULTILEVEL CONTROL OF EXTRACELLULAR SUCROSE METABOLISM IN *STREPTOCOCCUS SALIVARIUS*

BY

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In order to study the role of sucrose in the regulation and control of its metabolism by *Streptococcus salivarius*, standardized experimental conditions were established. It was found that *de novo* synthesis was required for the production of extracellular glucosyltransferase activity which, upon the addition of sucrose, became associated with the cell surface. Conversely, cell-associated fructosyltransferase activity required genetic induction for production and cell-surface association, but required sucrose for release from the surface framework. Extracellular fructanase activity was twofold higher when cells were grown in sucrose than when cells were grown in other sugars. This increase occurred within 5 minutes but was diminished by rifampicin and chloramphenicol. The extracellular dextranase activity of cells grown in sucrose was tenfold higher than that of cells grown in other sugars. This activity increased within 5 minutes following the addition of sucrose to galactose-grown cells and was affected

by neither rifampicin nor chloramphenicol. Dextranase appeared to be tightly controlled by a dextranase inhibitor that was displaced by sucrose, and by at least one other factor, that regulated or directed hydrolysis of dextran substrates.

To analyze the function and regulation of the extracellular endodextranase (α-1,6-glucan hydrolase, EC 3.2.1.11) from *S. salivarius*, a molecular approach was taken. Dextranase-positive recombinants from a genomic library were identified by their ability to clear zones in blue dextran-containing agar. One clone, PD1, contained a promoter element and structural gene which encoded an active 190 kilodalton protein. The native dextranase had an apparent molecular mass of 110 kilodaltons. *Streptococcus salivarius* contained a single copy of the endodextranase gene. Product analysis of dextranase activity on various substrates by thin layer chromatography revealed the expected isomaltosaccharides produced by the recombinant enzyme, but was unable to resolve the larger polysaccharide products of the native supernatant. *Streptococcus salivarius* utilized neither substrates nor products of dextran hydrolysis for growth, suggesting that dextranase has a synthetic role in this organism. The versatility in both genetic and biochemical control mechanisms, of this complex set of enzymes allows tight regulation and quick response to environmental stimuli, a potentially invaluable characteristic for an inhabitant of the oral cavity.

CHAPTER 1
INTRODUCTION

Streptococcus salivarius is a Gram-positive coccoid bacterium which is capable of growing in short or long chains (Hardie, 1986). This organism grows in the presence of oxygen and can be found in the oral cavity of humans and animals where it is primarily associated with the tongue and saliva, but also can be isolated from fecal material. Some strains have been linked to infectious endocarditis and dental caries in gnotobiotic rats (Hardie, 1986).

It also has been shown that a significant number of *S. salivarius* isolates have the ability to adhere to other structures in the oral cavity besides epithelial surfaces, namely, the pellicle on the teeth (Weerkamp & McBride, 1980). *Streptococcus salivarius* may be a primary colonizer, providing an initial attachment site for more fastidious, less oxygen-tolerant organisms. As *S. salivarius* is able to attach to tooth surfaces, but is found in low numbers in dental plaque (Weerkamp & McBride, 1980), it has been suggested that some adherence capabilities are rarely or never used. Furthermore, the ability of *S. salivarius* to adhere may require protease-sensitive components (Weerkamp & McBride, 1980) and may also involve covalent linkage to the peptidoglycan structure (Weerkamp & Jacobs, 1982).

Streptococcus salivarius is a successful inhabitant of the human oral cavity. In addition, it is capable of forming aggregates with several oral anaerobic, Gram-negative bacteria (Gibbons & Nygaard, 1970; Weerkamp

& McBride, 1981) and may form the basis for their inital colonization. The ability to attach to host surfaces and to adhere to other bacteria may be mediated by surface appendages associated with a fibrillar layer, termed the "fuzzy coat." This extracellular layer is present on *S. salivarius* (Gibbons *et al.*, 1972) and other streptococci, such as *Streptococcus pyogenes* (Ellen & Gibbons, 1972). Two morphologically distinct types of fibrils and several factors mediating attachment to host surfaces and other oral bacteria (Weerkamp & McBride, 1981) are found on the surface of *S. salivarius* (Handley *et al.*, 1984).

The role of sucrose-derived extracellular polysaccharides synthesized by oral streptococci in cariogenicity, adherence, and colonization has been well established (Hamada & Slade, 1980). Extracellular enzymes of *S. salivarius* involved in sucrose metabolism include: glucosyltransferases (GTFs; EC 2.4.1.10), which catalyze the synthesis of glucans from sucrose; fructosyltransferases (FTFs; EC 2.3.1.10), which catalyze the synthesis of fructans, either levan or inulin, from sucrose; dextranase (α-1,6-glucan hydrolase; EC 3.2.1.11), which partially degrades glucan; and fructanase (β-D-fructan fructohydrolase; EC 3.2.1.80), which releases free fructose by hydrolyzing fructan polymers.

These enzymes are part of a very complex system including catabolic enzymes which break down not only sucrose, but products derived from sucrose, such as dextran, levan, and inulin (Chassy *et al.*, 1974; Hamada *et al.*, 1975; Schachtele *et al.*, 1975b; Russell, 1979; Takahashi *et al.*, 1985; Burne *et al.*, 1987) (Figure 1-1). There are also anabolic enzymes which catalyze the synthesis of products using sucrose or sucrose-derived substrates (Slee & Tanzer, 1980; Hamada & Slade, 1980; Furata *et al.*, 1985). These processes occur both intracellularly and extracellularly and,

in the former case, require specialized transport systems for sucrose and sucrose-derived products (Ellwood & Hamilton, 1982; Postma & Lengeler, 1985; Lodge & Jacobson, 1988; Reizer *et al.*, 1988; Russell & Ferretti, 1990).

Despite the large amount of information available pertaining to the α-D-glucosyltransferases, there are still many fundamental questions which remain unanswered. It has been difficult to make valid comparisons between the different GTFs because their properties depend on the strain producing them, the isolation procedures used, growth conditions and the number of GTFs produced (Montville *et al.*, 1977). In fact, the exact number and nature of GTFs required for glucan synthesis is still controversial. A number of enzymes tend to form aggregates which include invertase, FTF, dextranase, uncharacterized proteins, as well as lipids, peptidoglycan, teichoic acids, and carbohydrates (Montville *et al.*, 1977), making isolation extremely difficult. It is reasonable to assume that many studies have been performed with non-homogeneous fractions and that inaccurate conclusions have been drawn. The analysis of the glucan products of these enzymes, i.e., the determination of the glycosidic linkages, the distribution of branches, and molecular weights has been equally difficult (Walker *et al.*, 1988). Since growth conditions and rates determine the characteristics of the glucan produced, the ratio of α–(1,3) to α–(1,6) glycosidic bonds, and consequently the solubility properties of the dextran product, vary accordingly (Walker & Jacques, 1987). It has not been possible, even with recombinant DNA techniques, to clarify the relationship between individual enzymes and the type of linkage synthesis they catalyze or the cooperation between multiple forms of glucosyltransferases.

Even less progress has been made in the characterization of the β-D-fructosyltransferases and the fructans they produce. The levan produced by *S. salivarius* has been characterized as a levan possessing β-(2,6) linkages with β-(2,1) branchpoints (Hancock *et al.*, 1976). It is thought that a single FTF is capable of synthesizing both β-(2,6) and β-(2,1)-fructans (Jacques *et al.*, 1985b). The relative amount of fructan to dextran in plaque and saliva is small, probably due to rapid hydrolysis of the former offsetting its greater rate of synthesis (Wood, 1969). Although *S. salivarius* makes large amounts of fructan, it also produces a β-D-fructan fructohydrolase (Burne *et al.*, 1987) which hydrolyzes the levan polymer to free fructose, which in turn is catabolized mainly to lactic acid.

Bacteria have evolved mechanisms by which full advantage is made of exogenously supplied nutrients, even though entry of these nutrients into the cell is not without cost. Virtually all biosynthetic pathways, polymerization reactions, and assembly processes require coordination if orderly growth is to occur. Control of such metabolic pathways can be established by regulating the kinds and amounts of macromolecules made, by control of gene expression and subsequent control of enzyme activity. Modulation of enzymatic activity by inhibition or activation of allosteric proteins by their specific effectors and by substrate/product concentrations is best understood (Ingraham *et al.*, 1983).

Until recently, the enzymes involved in sucrose metabolism in oral streptococci had been assumed to be constitutive as they are produced in the absence of substrate (Janda & Kuramitsu, 1978; Wenham *et al.*, 1979). Although there have been suggestions in the literature that sucrose metabolizing enzymes may be under some form of regulation (Keevil *et al.*, 1983; Walker *et al.*, 1983; Walker & Jacques, 1987), it has been

difficult to show this conclusively due to the difficulties in isolating individual enzymes, variation between strains, differences in enzyme production due to growth conditions, and the general complexity in the network of these and other enzymes which metabolize sucrose. Understanding the regulation and control of enzymes involved in sucrose metabolism will be necessary for an in-depth analysis of the role of this organism in oral pathology.

Work presented in this dissertation shows that this organism uses a variety of mechanisms to control this set of enzymes, allowing the regulation of their expression and function at several widely separated stages in their production. A first step in establishing whether or not these enzymes are under any form of regulation was to determine the role of sucrose in the control of its metabolism. Once this was accomplished, the scope of the study was narrowed to one enzyme, dextranase.

A major goal of this study was to determine the contribution of dextranase to the physiology of *S. salivarius*. It was important to distinguish between two proposals: 1) that the primary function of dextranase, although a hydrolytic enzyme by definition, is, in fact to act as a synthetic catalyst; or 2) that its major contribution is the degradation of large glucans to smaller polymers serving as substrates for catabolism.

In a putative biosynthetic capacity, it is possible that dextranase could serve to provide primer or branchpoints for the glucosyltransferases (Walker, 1972; Germaine *et al.*, 1977; Felgenhauer & Trautner, 1983). The ratio of α-(1,3)- to α-(1,6)-glycosidic linkages and therefore the solubility properties of dextran made by *S. salivarius* and other organisms may be altered, if involvement in glucan formation is a major function of dextranase. Since the degree of solubility of glucans has been shown to be

an important factor in colonization, plaque formation, and caries development, dextranase then would have a great potential for impacting on these processes (Walker & Jacques, 1987).

Alternatively, if dextranase were acting in a catabolic pathway, to break down dextran eventually to yield glucose, it would be acting as an antagonist to the extracellular GTFs, thus explaining the low apparent levels of GTF activity (determined by the amount of dextran produced) in this organism (Hamada *et al.*, 1975; Schachtele *et al.*, 1975). If this were the case, the presence of dextranase would obscure any conclusions made in studies of GTF in any organism producing both these enzymatic activities and consequently, any reaction influenced by dextran, i.e., adherence, aggregation, and the distribution of GTFs between cell-surfaces and culture supernatants (Walker *et al.*, 1981).

Extracellular endodextranase hydrolyzes large dextran molecules to smaller polymers, predominantly oligoisomaltosaccharides (Dewar & Walker, 1975). These short isomaltosaccharides should be readily transported across the cell membrane, where a second enzyme, such as the intracellular exodextranase described for *Streptococcus mutans* (Burne *et al.*, 1986; Russell & Ferretti, 1990), would hydrolyze them completely to glucose. The tandem action of the endo- and exo-dextranases would be an energy efficient system for the rapid hydrolysis and utilization of dextran. If this were true, the extracellular endodextranase of *S. salivarius* could play a key catabolic role in the physiology of this species and enable it to use the dextran product of its own GTFs and/or those produced by other oral organisms as short term energy reservoirs. Parker and Creamer (1971) demonstrated that polysaccharides could serve as significant

reserves and that some glucans could support growth of some oral streptococci.

Considering the fact that dextranase is produced by many strains of oral streptococci (Dewar & Walker, 1975; Schachtele *et al.*, 1975a; Ellis & Miller, 1977; Felgenhauer & Trautner, 1983), it is suprising how little is known about this enzyme. Most information to date on dextranase in oral streptococci has been obtained from *Streptococcus sobrinus* (Barrett *et al.*, 1986; Walker *et al.*, 1988). The preliminary studies presented in this dissertation are the first dealing with dextranase from *S. salivarius*. Barrett *et al.* (1987) developed a purification scheme for dextranase present in *S. sobrinus* culture supernatant fluids. These investigators recovered the majority of the dextranase in two forms, having molecular weights of 175 KD and 160 KD. The lower molecular weight form was thought to be a proteolytic breakdown product of the 175 KD dextranase. This same group (Wanda, 1990) reported the molecular mass of the native enzyme to be 280 KD - 200 KD, by gel filtration. When *S. sobrinus* gene libraries were screened for dextranase activity, three phenotypes of recombinant clones were identified. A *Pst* I contiguous library produced a 175 KD dextranase, a *Sau* 3A noncontiguous library produced a 150 KD dextranase, and an *Eco* RI noncontiguous library produced both dextranases (Barrett *et al.*, 1987). The variation in molecular weight was ascribed to either deletions of C-terminal ends in the noncontiguous libraries, "scrambling" of the dextranase structural gene, or the cloning of two separate genes. The authors believe the latter is unlikely due to genetic characterizations of *S. sobrinus* dextranase mutants which suggested a single dextranase gene. The clone from the *Sau* 3A library was chosen for further study. The dextranase from this construct was determined to be a

periplasmic enzyme in *E. coli* with a molecular weight ranging from 140 KD to 80 KD (by SDS-PAGE with blue dextran) (Wanda, 1990). Several subclones were obtained by a partial digestion with *Pvu* II and religation. The majority of the *dex* gene was found to reside on a 2.6 kb *Pvu* II fragment; however, this fragment did not contain the *dex* promoter. Attempts to obtain sequence of the *dex* gene of *S. sobrinus* have not been successful to this point (R. Curtiss III, personal communication).

The following chapters show that extracellular dextranase activity was higher in sucrose-grown *S. salivarius* PC-1 cells compared to cells grown in glucose, fructose, or galactose (Figure 2-2); that dextranase activity increased 100-fold when sucrose was added to cells growing in galactose (Figure 2-3) and that this increase was not affected by transcriptional or translational inhibitors (Table 2-1); that dextranase was active when cells were grown in sucrose and inactive in galactose-grown cells (Figure 3-5a); and that sucrose added to cell-free *S. salivarius* CDM/galactose culture supernatant activated the native dextranase (Figure 3-5b). These results suggest that activation of native dextranase by sucrose may involve the displacement of a dextranase inhibitor.

Hamelik and McCabe (1982) concluded that the presence of an inhibitor in batch-grown culture fluids of *S. mutans* accounted for the absence of endodextranase activity in strains known to produce this enzyme. This finding was in direct conflict with earlier conclusions that endodextranase production was growth-dependent and that the enzyme was labile at pH 5.0 (Walker *et al.*, 1981). Sun *et al.* (1990) have cloned and sequenced the dextranase inhibitor gene (*dei*) from *S. sobrinus*. The *dei* gene specifies a 330 amino acid protein with a molecular weight of approximately 36 KD and carries its own promoter. DNA from serotypes

a,d, and *g* of mutans streptococci was recognized by Southern hybridization using the *dei* probe. The widespread distribution of a dextranase inhibitor (Sun *et al.*, 1990) may represent a common theme of postgenetic regulation of this enzyme, making this a feasible model for studying enzyme regulation in oral streptococci. The apparent presence of such an inhibitor in *S. salivarius* is explored further in Chapter 3.

Since *S. salivarius* is a common inhabitant of the oral cavity, is among the earliest colonizers after birth, and constitutes a major proportion of the streptococci found on soft tissue in the mouth, it is likely that any extracellular enzyme produced by this organism would have a profound effect on the oral ecology. All indications are that dextranase has the ability to participate in plaque formation and modification through its effects on the polysaccharides thought to be important in bacterial aggregation and adherence (Gibbons & Van Houte, 1975). Dextranase may act directly in conjunction with GTFs in the modification of dextran polymers, by providing primer or branchpoints and thus may be involved in determining the degree of dextran solubility. This enzyme may also break down the accumulated dextran in plaque, destabilizing the plaque matrix and at the same time providing glucose for consumption by plaque organisms. It also is conceivable, as previously suggested, that dextranase may be important in the physiology and growth of *S. salivarius*, giving this species a competitive edge in the oral environment.

The fact that dietary sucrose would serve to activate dextranase is of considerable interest since it is under these nutritional conditions that dental caries has been shown to occur. This is especially true since the ability to make dextran has been held responsible for allowing colonization by cariogenic organisms. An alternative explanation for the correlation

between sucrose and caries may be that dextranase provides additional substrate for acid production as it breaks down the stored dextran. To test this hypothesis further, the polysaccharides which serve as substrates for dextranase were determined. The ability of *S. salivarius* to grow in dextran or possible products of glucan hydrolysis also was established. The results of these studies are presented in Chapter 3.

Proteins which bind glucans and enzymes which are involved in the metabolism of glucans contain glucan binding regions (Ferretti *et al.*, 1988; Sato & Kuramitsu, 1988; Uedo *et al.*, 1988; Sato *et al.*, 1989; Banas *et al.*, 1990). It would be of interest to compare the nucleotide and amino acid sequences of the *dex* gene to sequences of other such proteins. This should allow the division of this protein into functional domains, i.e., glucan binding and catalytic regions. The DNA sequence should also give specific information about initiation and termination signals, promoter and coding regions, and a more accurate estimate of the molecular weight of dextranase. To accomplish this, it was first necessary to clone and characterize the *S. salivarius dex* gene and its product. This information is found in Chapters 3 and 4.

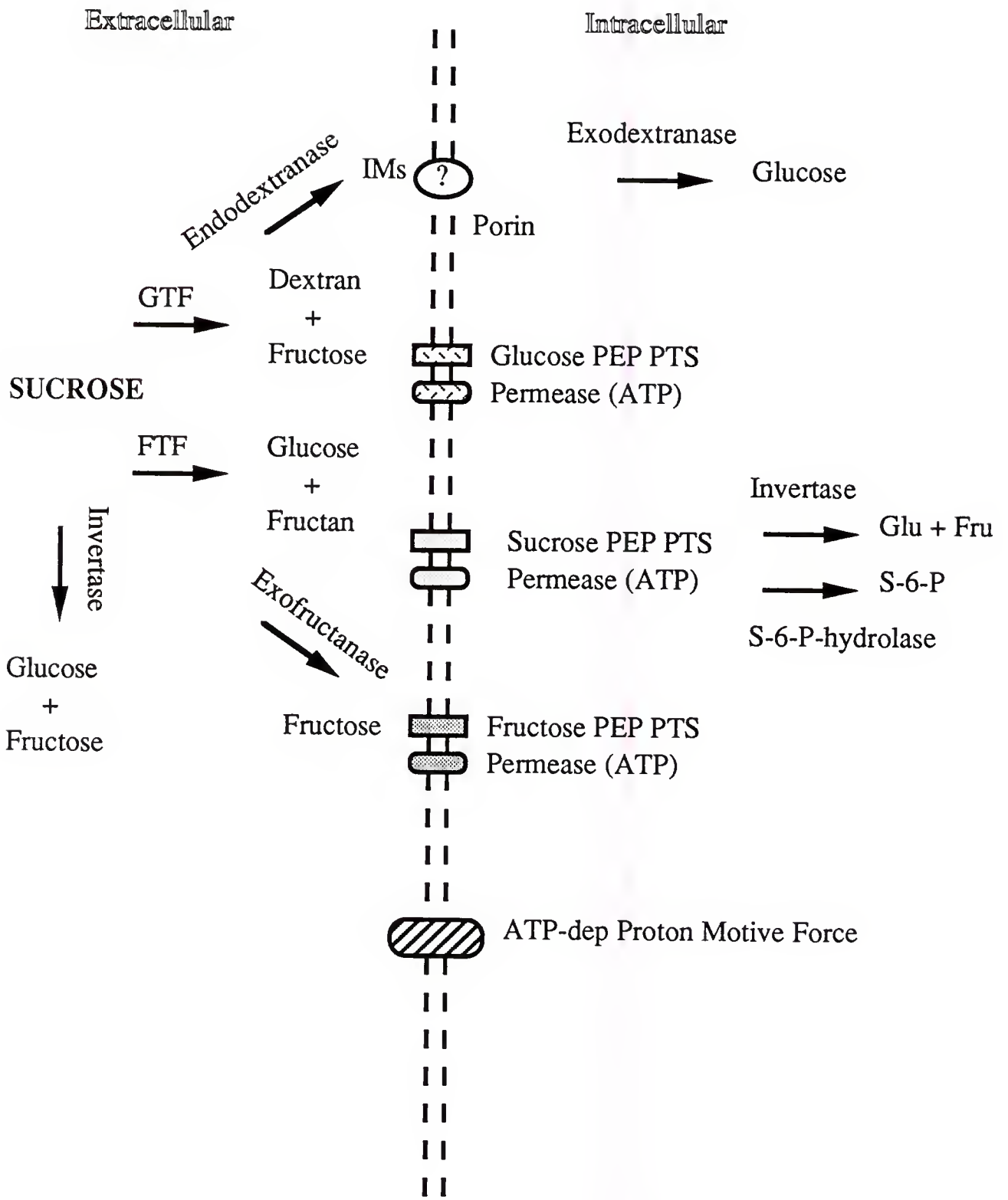


Figure 1-1. Diagramatic representation of sucrose metabolism in *S. salivarius*.

CHAPTER 2
MULTILEVEL CONTROL OF EXTRACELLULAR SUCROSE METABOLISM IN *STREPTOCOCCUS SALIVARIUS* BY SUCROSE

Introduction

Sucrose has a marked impact on the microbial ecology of the oral cavity. This common disaccharide enhances plaque development and is associated with dental caries (de Stoppelaar *et al.*, 1970; Carlsson & Johansson, 1973; Gibbons & van Houte, 1973; Hamada & Slade, 1980). Streptococcal enzymes which metabolize sucrose, some to synthesize polysaccharides important in microbial adhesion or cohesion (Gibbons & van Houte, 1975; Wenham *et al.*, 1981; Koga *et al.*, 1986), and others to hydrolyze these polysaccharide products, have been shown to be virulence factors in the mutans group of oral streptococci (Curtiss *et al.*, 1987; Sato *et al.*, 1987; Schroeder *et al.*, 1989). However, little has been done to study analogous enzymes in *Streptococcus salivarius*, a major inhabitant of the soft tissues of the oral cavity.

The complex system of extracellular enzymes involved in sucrose metabolism include glucosyltransferases (GTFs; EC 2.4.1.5), which catalyze the synthesis of glucans from sucrose; fructosyltransferases (FTFs; EC 2.4.1.10), which catalyze the synthesis of fructans, either levan or inulin, from sucrose; dextranase (α-1,6-glucan hydrolase, EC 3.2.1.11), which partially degrades glucan; and fructanase (β-D-fructan

fructohydrolase, EC 3.2.1.80), which releases free fructose by hydrolyzing fructan polymers. Since these enzymes are produced in the absence of substrate, they have been assumed to be constitutive in oral streptococci (Janda & Kuramitsu, 1978; Wenham *et al.*, 1979). Although there have been suggestions in the literature that this might not be the case (Keevil *et al.*, 1983; Walker *et al.*, 1983; Walker & Jacques, 1987), it has been difficult to show conclusively that these enzymes are under any kind of regulatory control. This has been due to difficulties in isolating individual enzymes, variation between strains, differences in enzyme production due to growth conditions, and the general complexity in the network of these and other enzymes which metabolize sucrose.

Streptococcus salivarius is a common inhabitant of the oral cavity, colonizing preferably the tongue dorsum and buccal epithelium (Weerkamp & McBride, 1980) as well as being found in the saliva. Although it is not known to be a major oral pathogen, this species possesses a variety of anabolic (i.e. GTF, FTF) and catabolic (i.e. dextranase, fructanase) extracellular enzymes associated with sucrose metabolism (Chassy *et al.*, 1976; Takahashi *et al.*, 1983; Houck *et al.*, 1987). Studies described below indicate that sucrose plays a role in the regulation of its metabolism in this microbial species and that significant versatility is maintained in the mechanisms by which this is accomplished (Townsend & Bleiweis, 1989).

Methods

Bacterial strain and growth conditions.

A fresh isolate of *S. salivarius* was used in these studies. This isolate, designated PC-1, was isolated in this laboratory. Subculturing was kept to a minimum by using stock cultures maintained at -70°C in 25% (v/v) glycerol.

A chemically defined medium (CDM), prepared according to Terleckyj *et al.* (1975) was used for bacterial growth in all experiments described in this paper. The energy source (sugar) varied depending on the experimental conditions as described in Results. Reagent grade sugars were purchased from Sigma Chemical Co. All cultures were grown at 37°C.

Cell growth, harvest and preparation.

Growth curves were generated by inoculating CDM or CDM/sugar (glucose, fructose, galactose, glucosamine, or sucrose) (10 mM) with a CDM/glucose (10 mM) starter culture at a 1:10 ratio. A Klett-Summerson photoelectric colorimeter, with a No. 54 filter, was used to take optical density measurements every 30 minutes until stationary phase was reached. Each data point in Figure 2-1 represents the mean of triplicate samples from a representative experiment. The pH of each culture was determined at the same time intervals using colorpHast™ indicator sticks (MC/B Manufacturing Chemists, Inc.).

To establish the basal level of the enzymatic activities in *S. salivarius* cells, a CDM/glucose (10 mM) starter culture was added at a ratio of 1:10

to CDM/glucose, fructose, galactose, or sucrose. When cultures reached the desired density (Klett 75, mid-exponential phase) the cells were harvested by filtration using a 0.2 mm membrane filter (Gelman Sciences, Inc.), washed once with 50 mM potassium phosphate buffer, pH 6.35 (the buffer used throughout this study), collected by centrifugation (4000*g*, 20°C, 5 minutes), and then resuspended to a tenfold concentration equivalent in the same buffer containing 10 mM NaF (McCabe & Smith, 1975). The cells were held at 4°C until enzymatic assays were performed (within 24 hours). The supernatants from these cultures were concentrated immediately using Centriprep™ concentrators (Amicon) at 3000*g*, dialyzed overnight and reconstituted to a tenfold concentration equivalent in the potassium phosphate buffer. The supernatants were assayed for enzymatic activity after dialysis.

For sucrose shift assays, a PC-1 starter culture, grown as above, was added to CDM/galactose. This culture was grown to a Klett reading of 75. At this time sucrose was added to a final concentration of 10 mM and aliquots were taken over time as indicated in Figures 2-3 and 2-6. The cells and culture fluids then were harvested and concentrated as above. Aliquots of cells collected from CDM/galactose cultures also were washed vigorously two times with 0.04% SDS and several concentrations of Tween 80 (0.02, 0.04, 0.06%), and twice with 50 mM potassium phosphate buffer, pH 6.35. The cells were harvested by centrifugation (4000*g*, 20°C, 5 minutes) after each wash, finally resuspended to a tenfold concentration equivalent in the same buffer containing 10 mM NaF, and assayed for retention of GTF and FTF activities.

Antibiotic inhibition studies were conducted in a manner similar to the sucrose shift assays. A starter culture was added to CDM/galactose in

the proportions given above and grown to the same optical density. At this time, sucrose (10 mM), chloramphenicol (100 mg ml^{-1}), rifampicin (100 mg ml^{-1}), or combinations of these were added to aliquots of the culture and then incubated at 37°C for 1 hour. The cells and supernatants were treated as above prior to enzyme assays. Control cultures contained none of these reagents.

Assay of enzyme activities.

Dextranase and fructanase activities were determined using a standard assay for reducing sugars designed by Somogyi (1951) and Nelson (1944). Substrate, either dextran Type 100C (Sigma Chemical Co.) or levan from *Aerobacter levanicum* (Sigma Chemical Co.) at a final concentration of 0.25 mg ml^{-1} was incubated with cells or supernatant as prepared above. Reactions were stopped after 180 minutes. The amount of reducing sugar released (glucose or fructose depending on the substrate) was determined by comparison with glucose or fructose standards. One unit of enzyme activity (U) was defined as the amount of dextranase or fructanase catalyzing the release of 1 mmol reducing sugar (glucose or fructose) min^{-1} ml^{-1}. Each data point shows the mean (+/- SD) of triplicate samples from a representative experiment.

GTF and FTF activities were quantitated by a modification of the standard procedure developed by Robrish *et al.*, (1972) where sucrose, labeled either in the glucose or fructose moiety, was incorporated into ethanol-precipitable polymer. Reaction mixtures consisted of tenfold concentrated sample (cells or supernatant), 1 mM sucrose carrier, 0.25 mg ml^{-1} commercially obtained dextran or levan primer, and labeled sucrose,

[glucose-^{14}C(U), 7.7 x 10^{-5} mmol ml^{-1}, at 261.0 mCi $mmol^{-1}$] or [fructose-^{14}C(U), 7.5 x 10^{-5} mmol ml^{-1}, at 267.0 mCi $mmol^{-1}$] (NEN Research Products). Reactions were carried out in a total volume of 100 µl in 12 x 75 mm borosilicate disposable culture tubes at 37°C for 90 minutes. The polymer was precipitated with 300 ml ice-cold 95% (v/v) ethanol for at least 30 minutes. The ethanol insoluble product was collected on 25 mm extra thick glass fiber filters (Gelman Sciences, Inc.) cut to fit disposable Microfolds™ (V & P Scientific, Inc.). The microfold system is a 96 chamber micro-plate with a small opening in the bottom of each well. The filter rests on top of the opening and immobilizes the precipitate when vacuum is applied to the bottom of the manifold. Each filter was washed once with ice-cold 95% (v/v) ethanol and air-dried before being placed in vials with 3 ml aqueous counting scintillant (ACS) (Amersham). Each sample was counted for 5 minutes in a Beckman LS 3801 Liquid Scintillation System. The adaptation of this procedure to a microassay system made it possible to handle multiple samples with greater accuracy and reproducibility. One unit of enzyme activity (U) was defined as the amount of GTF or FTF that catalyzed the incorporation of 1 nmol of the glucose or fructose moeity of labeled sucrose min^{-1} ml^{-1}. Each data point represents the mean (+/- SD) of triplicates from a representative experiment. Depending on experimental conditions employed, 65-95% of the polymer collected from GTF activities and 90% of the fructan synthesized in the FTF assays was water-soluble. No attempt was made to correlate these properties with regulatory effects as that distinction was beyond the limits of these assays. Therefore, only the total amount of polymer produced was reported.

Results

Carbon source utilization of *S. salivarius* PC-1.

In order to develop appropriate culture conditions, *S. salivarius* PC-1, was grown in a number of carbohydrate carbon sources (Figure 2-1). When cells were grown in glucose the doubling time at mid-exponential phase was 49 minutes. It was determined that the growth rates at mid-exponential phase were similar for cells grown in either fructose (60 minutes), sucrose (60 minutes), or galactose (62 minutes); however, the lag time for fructose-grown cells was longer than for the other cultures. Cells did not grow in glucosamine. Cells taken at a Klett reading of 75 were at mid-exponential phase regardless of the carbon source. This allowed the normalization of all subsequent experiments to this value, and the use of galactose as a "non-sucrose related" carbon source in sucrose shift assays. Cultures grown in the various sugars and harvested at an optical density of Klett 75 had a range of pH values between 6.1 and 6.4. Over the duration of the growth experiments, all cultures shifted from an initial pH of 7.0 to a final pH of 4.0 when glucose, galactose, fructose, or sucrose was used as the growth substrate.

Dextranase and fructanase activities of *S. salivarius* PC-1.

Cultures of PC-1 were grown in 10mM glucose, fructose, galactose, or sucrose, and the dextranase and fructanase activities of cells and culture fluids were measured. Under all conditions tested, cell-associated activity for these enzymes was not detected indicating that both dextranase and fructanase are extracellular enzymes. If an intracellular dextranase exists

in this organism, as in related streptococci (Dewar & Walker, 1975; Walker *et al.*, 1980; Walker *et al.*, 1981; Russell & Ferretti, 1990), its activity was not measurable under these conditions. Dextranase levels were similar whether the cells were grown in glucose, fructose, or galactose; the same held true for fructanase (levanase) levels (Figure 2-2). Growth in sucrose; however, resulted in a twofold higher fructanase activity and a tenfold higher dextranase activity. From these preliminary experiments it appeared that growth in sucrose resulted in significantly increased levels of both dextranase and fructanase activities in culture supernatants.

Effects of sucrose shift on dextranase and fructanase activities.

Having noted the effect that growth in sucrose had on the production of both dextranase and fructanase, it was of interest to measure the response of these enzymatic activities to the addition of sucrose to cells growing in galactose (Figure 2-3). The addition of sucrose resulted in an immediate (< 5 minutes) increase in both dextranase and fructanase activities followed by a period of stabilization. While fructanase levels increased by 64% upon the addition of sucrose, dextranase levels increased more than 100-fold.

Effect of antibiotics on dextranase and fructanase production.

To determine whether the sucrose effect described above was being implemented at the genetic level, previously determined inhibitory concentrations of antibiotics specifically targeted to transcription (rifampicin) or translation (chloramphenicol) were tested. In the absence of sucrose, dextranase levels in culture fluids remained very low whether

the cells were untreated, or treated with chloramphenicol or rifampicin (Table 2-1). However, there was a significant increase (100-fold) in dextranase activity upon the addition of sucrose, even in the presence of chloramphenicol or rifampicin. It appears, therefore, that the increases in dextranase activity observed in these experiments do not reflect genetic induction, and that regulation at the post-translational level is likely. Fructanase activity measured under the conditions described was relatively high whether the cells were grown in glucose, fructose, or galactose (Figure 2-2); therefore, the net increase upon the addition of sucrose was not as dramatic as that seen with dextranase activity (Figure 2-3). Furthermore, unlike dextranase, this increase was reduced significantly, from 78% with sucrose alone to 47% and 33%, respectively, by transcriptional and translational inhibitors (Table 2-1), indicating a possible genetic induction.

GTF and FTF activities of *S. salivarius* PC-1.

These experiments were performed with and without a dextran or levan primer for GTF or FTF respectively. The addition of primer increased the incorporation of radiolabeled glucose in the GTF assays by approximately 15-25% and the incorporation of radiolabeled fructose in the FTF assay by 10-15%. Data presented here are from experiments that include primer.

The measurable activities of both cell-associated or released GTF were quite low. This probably was due to the presence of dextranase which was able to solubilize the radiolabeled products. Supporting this theory was the apparent inverse relationship between the amount of GTF

activity (Figure 2-4) and dextranase activity (Figure 2-2) measured under these conditions. Cell-associated GTF activity was similar in cells grown on sucrose, galactose, or glucose, and higher in cells grown on fructose (Figure 2-4). Glucose-grown cells produced the highest apparent activity of the secreted form of GTF as compared to fructose-, sucrose-, or galactose-grown cells.

Cell-associated FTF activity was greater than extracellular activity when cells were grown in glucose, fructose, or galactose (Figure 2-5). However, soluble FTF activity increased when cells were grown on sucrose. The total FTF activity for sucrose-grown cultures was about twofold greater than for galactose-grown cultures (Figure 2-5).

Effect of sucrose shift on GTF and FTF activities.

When sucrose was added to cells growing in galactose, cell-associated GTF activity appeared to increase initially, but remained low (Figure 2-6a). In contrast, released activity showed an initial decline before returning to original values, suggesting a sucrose-mediated, cell-association of secreted GTF. As seen in Figure 2-5, the majority of FTF activity was cell-associated when the cells were grown in galactose. However, within 5 minutes after the addition of sucrose, there was a complete reversal of the location of FTF activity (Figure 2-6b). This translocation effect was immediate and profound.

To investigate the nature of the cell-surface interactions of these enzymes, galactose-grown cells were subjected to a number of different washing procedures and then assayed for GTF and FTF activities. The 0.04% SDS wash removed 95% of cell-associated GTF activity, but

reduced cell-associated FTF by only 34%. No FTF activity was dissociated from the cell surface by Tween 80 at any concentration, whereas cell-associated GTF activity was reduced 57% by 0.06% Tween 80, 71% by 0.04% Tween, and 81% by 0.02% Tween.

It became important to determine if there was genetic induction following the addition of sucrose that might account for the ultimate increase in total GTF and FTF activities over time.

Effect of antibiotics on GTF and FTF production.

An inhibition experiment similar to that described for dextranase and fructanase (Table 2-1) was performed for the glycosyltransferases. The untreated control and rifampicin-, or chloramphenicol-treated cells exhibited levels of cell-associated GTF which were less than those of the extracellular form of this enzyme (Table 2-2). The total GTF activity in response to each of these treatments was very similar, although rifampicin treatment caused an unexpected release of GTF activity into the fluid phase. Upon the addition of sucrose, extracellular activity remained constant while cell-associated activity increased almost fourfold. The total GTF activity in the presence of sucrose was twofold higher than in the absence of sucrose. Transcriptional and translational inhibitors, in conjunction with sucrose; however, decreased the amount of extracellular GTF activity by 69-71% when compared with untreated control and sucrose-exposed cultures. The effects of rifampicin and chloramphenicol may be due to the inhibition of all cellular protein synthesis.

The cell-associated form of FTF predominated in galactose-grown, and in rifampicin- and chloramphenicol-treated cells; however, upon the

addition of sucrose, extracellular FTF activity increased almost sevenfold while cell-associated activity remained unchanged (Table 2-3). When chloramphenicol or rifampicin was added together with sucrose, extracellular FTF levels remained constant, but cell-associated FTF activity decreased sharply (71-73%). Total FTF activity was highest when sucrose was added without chloramphenicol or rifampicin. Taken in aggregate, these data indicate both genetic and posttranslational regulation of FTF by its substrate sucrose.

Discussion

In studies such as those presented above, differential enzyme production due to growth phase variations or enzyme degradation in aging cultures must be avoided. Growth experiments; therefore, were run extensively to determine the optimum stage at which cells grew at a consistent rate without exhausting nutrients. This made it possible to normalize all experiments by growing the cells to a predetermined point in mid-exponential phase regardless of further treatment. The use of single sugars as sole carbon sources in a chemically-defined medium minimized the effects of nutrition which could complicate interpretation of results. Fortunately, *S. salivarius* PC-1 utilized galactose at a rate similar to that of sucrose and other sugars. This allowed the use of galactose as a "non-sucrose related" carbon-source for the sucrose shift experiments. The pH levels (6.1-6.4) of *S. salivarius* PC-1 cultures grown to an optical density of Klett 75, regardless of the sugar used, were well within the ranges determined by others for optimal GTF (McCabe & Smith, 1973),

dextranase (Walker *et al.*, 1980), FTF (Wenham *et al.*, 1979), and fructanase (Jacques *et al.*, 1985b) production.

Cell preparation and treatment also were designed to be consistent within all experiments. This uniform approach provided cells at equivalent mass, growth phase, and concentration and allowed for the comparison of cell-associated and extracellular enzymatic activities under different experimental conditions. The enzymatic activities measured were taken to be an indication of the relative amounts of enzyme produced. The products assayed in the GTF and FTF experiments were the net result of synthesis (by GTF and FTF) and also degradation (by dextranase and fructanase). On the other hand, the substrate for GTF and FTF (sucrose) was not present in the dextranase and fructanase assays and therefore could not have contributed to net amount of product.

Chassy *et al.* (1976) found that the presence of sucrose reduced GTF activity in culture fluids of *S. salivarius*, but did not see a shift to the cell-associated form. These investigators were unable to distinguish between the possibilities of repression of GTF by sucrose or some modification in activity of constitutive GTF levels. In the present study, when sucrose was added to growing cells, GTF activity became associated with the cell surface (Figure 2-6a). The nature of this surface interaction is different from that observed with FTF, since GTF activity, but not FTF activity, can be washed from the surface with SDS and low concentrations of Tween 80.

Recently, Aduse-Opoku *et al.* (1989), have shown that the glucan-binding protein of *Streptococcus mutans* becomes bound to the cell surface on exposure to sucrose. If dextran were to form a bridge between the glucan binding domain of GTFs (Mooser & Wong, 1988) and these surface-bound glucan-binding lectins (Drake *et al.*, 1988; Banas *et al.*,

1990), washing with detergents may solubilize the dextran, releasing the GTF activity. McCabe and Smith (1973) reached similar conclusions concerning the binding of the GTFs of *S. mutans*. Another theory, proposed by others (Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978; Jacques *et al.*, 1985a) is that GTF synthesis and release are regulated by changes in the membrane fluidity determined by its fatty acid composition.

The *gtfB* and *gtfC* genes from *S. mutans* have been cloned (Aoki *et al.*, 1986; Pucci *et al.*, 1987; Hanada & Kuramitsu, 1988) and sequenced (Shiroza *et al.*, 1987; Ueda *et al.*, 1988). The nucleotide sequences designated the gene products as extracellular enzymes in that putative signal sequences were present and membrane anchor sequences were absent. This further supports the conclusion that cell-association of GTF activity is mediated by factors other than wall- or membrane-anchoring domains in the GTF molecule and that cell-associated and extracellular activities may be mediated by the same enzyme species.

Inhibitor studies (Table 2-2) and studies by other investigators (Janda & Kuramitsu, 1976; Montville *et al.*, 1977; Janda & Kuramitsu, 1978) indicate that *de novo* protein synthesis may be required for the synthesis of at least one extracellular GTF. Recently, Hudson and Curtiss (1990) showed an increase in the expression of *gtfB/C* from *S. mutans* in the presence of sucrose, thereby providing additional evidence of genetic regulation of this enzyme by its substrate in the oral streptococci. This production, followed by association of GTF activity to whole cells, may be part of a dynamic process involving glucan-binding lectins or still-unknown surface proteins. The changes in GTF activity seen upon the addition of sucrose are difficult to define biochemically due to multiple effects this molecule may have on the regulation (genetic induction),

location (cell-association), and measureable activity (antagonistic effects of dextranase) of this enzyme. Furthermore, each of these phenomena may differ for the individual GTFs in *S. salivarius* as recently described by Pitty *et al.* (1989). However, data presented here and by others (Janda & Kuramitsu, 1976; Montville *et al.*, 1977; Janda & Kuramitsu, 1978; Hudson & Curtiss, 1990) suggest that sucrose regulates the synthesis and distribution of GTF and that these two phenomena are independent of each other.

The gene encoding the *S. mutans* GS-5 FTF has been cloned (Sato & Kuramitsu, 1986) and sequenced (Shiroza & Kuramitsu, 1988). All indications have been that this enzyme, like GTF, is a secreted protein. Yet, Figure 2-6b and Table 2-3 clearly show that in the absence of sucrose the majority of FTF activity is found on the cell surface of *S. salivarius*, as previously demonstrated by Chassy *et al.* (1976). Several hypotheses regarding the location and activity of FTF have been formulated. It has been postulated that the cell-associated form of FTF is inactivated by proteolytic degradation (Jacques & Wittenberger, 1981), that FTF may be associated with the cytoplasmic membrane (Jacques, 1985), and that the lipid content of the membrane is responsible for the regulation and release of this enzyme (Pitty & Jacques, 1987). However, the suggestion can be made that the rapid release of this activity from the cell surface upon the addition of sucrose as seen in Figure 2-6b and Table 2-3, and recently confirmed by Milward and Jacques (1990), is due to the FTF's greater affinity for sucrose relative to its affinity for the surface framework to which it may be bound. This establishes a biological basis for the translocation phenomenon and also suggests that the distribution of FTF is regulated independently of its synthesis. Since the cell-associated FTF

activity cannot be washed from the cell surface with either ionic or non-ionic detergents it would argue against the enzyme being held through nonspecific hydrophilic or hydrophobic interactions, but would strongly suggest a more specific form of binding.

While a significant amount of the FTF activity can be recovered in the supernatant after the addition of sucrose, this does not fully account for the twofold increase in total activity (Table 2-3). Hudson and Curtiss (1990) have shown that in *S. mutans* such an effect may be due to increased transcriptional levels. Nucleotide sequence analysis has revealed regions of the *S. mutans* FTF gene which may be involved in its genetic regulation (Shiroza & Kuramitsu, 1988). *Streptococcus salivarius* also may regulate FTF production by substrate induction, but the sucrose-mediated release of FTF shown above clearly requires a surface-localized binding site for FTF. It is presently unclear whether this specialized binding protein must be synthesized and transported to the cell surface along with the FTF. However, data presented in Table 2-3 indicate *de novo* protein synthesis is associated, in some way, with the induction of cell-bound FTF by sucrose.

Streptococcus salivarius dextranase activity resides in the supernatant, away from the cell, and this activity increases at least 100-fold in the presence of sucrose. This is most likely not a genetic induction since neither chloramphenicol nor rifampicin affects dextranase production in the presence of sucrose. This should be compared with the effects of these inhibitors on fructanase (Table 2-1) where there is significant inhibition, indicating possible genetic induction. Sucrose may activate an otherwise inactive form of dextranase through some type of conformational change. However, since recombinant dextranase is active in the absence of sucrose (see Chapter 3), sucrose may act to release dextranase from a putative

dextranase inhibitor. Hamelik and McCabe (1982) have shown that in *Streptococcus sobrinus* the majority of dextranase is in a tightly bound, inactive, enzyme-inhibitor complex. An affector (presumably sucrose) with a high affinity for the inhibitor would be required for activation of dextranase.

Dextranase could act on the dextran polymer to provide primer or branch points for GTF (Walker, 1972; Germaine *et al.*, 1977). If involvement in glucan formation is a major function of dextranase, the ratio of α-(1,3) to α-(1,6) glycosidic linkages and therefore the solubility properties of dextran made by *S. salivarius* and other organisms may be altered. This would have a potential impact on colonization, plaque formation and caries development. Activation of dextranase by sucrose in order to modify dextran synthesis, therefore, is readily understandable.

Alternatively, dextranase could act at the beginning of a catabolic pathway resulting in the breakdown of dextran to eventually yield glucose (Hamada *et al.*, 1975b; Schachtele *et al.*, 1975). It is known that extracellular dextranase acts as an endoenzyme, cleaving the dextran into smaller polymers, predominantly isomaltosaccharides (Dewar & Walker, 1975). A regulatory role for sucrose in the catabolic function of dextranase is less obvious. However, from an energetic standpoint, the synthesis and degradation of glucans relies solely on bond energy. The short isomaltosaccharides released by the extracellular endodextranase should be readily transported across the cell membrane where an intracellular exodextranase, such as those recently cloned from *S. mutans* by Burne *et al.* (1986) and Russell and Ferretti (1990) would further degrade these short polymers to glucose. If this energy efficient system for obtaining glucose exists in *S. salivarius*, the extracellular endodextranase

could play a key catabolic role in the physiology of this species and regulation by sucrose would be understandable.

Furthermore, in this catabolic capacity, dextranase would be acting as an antagonist to the extracellular GTFs, thus explaining the apparent low levels of GTF activity in this organism. In fact, the presence of dextranase may obscure any conclusions made in studies of GTF in any organism producing both these enzymatic activities and consequently, any reaction influenced by dextran (aggregation, adherence, the distribution of GTFs between cell-surfaces and culture supernatants) (Walker *et al.*, 1981).

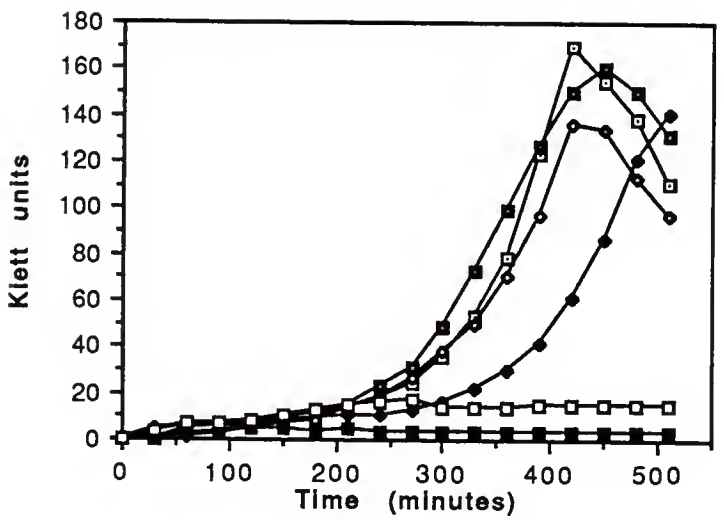


Figure 2-1. Carbon source utilization by *S. salivarius* PC-1

Cells were grown in CDM/glucose (10 mM) and aliquots transferred to either: CDM (open squares), CDM/glucosamine (closed squares), CDM/galactose (open diamonds), CDM/sucrose (dotted squares), CDM/fructose (closed diamonds), and CDM/glucose (solid dotted squares). Each sugar concentration was 10 mM, cells were incubated at 37°C, and Klett readings were taken as shown.

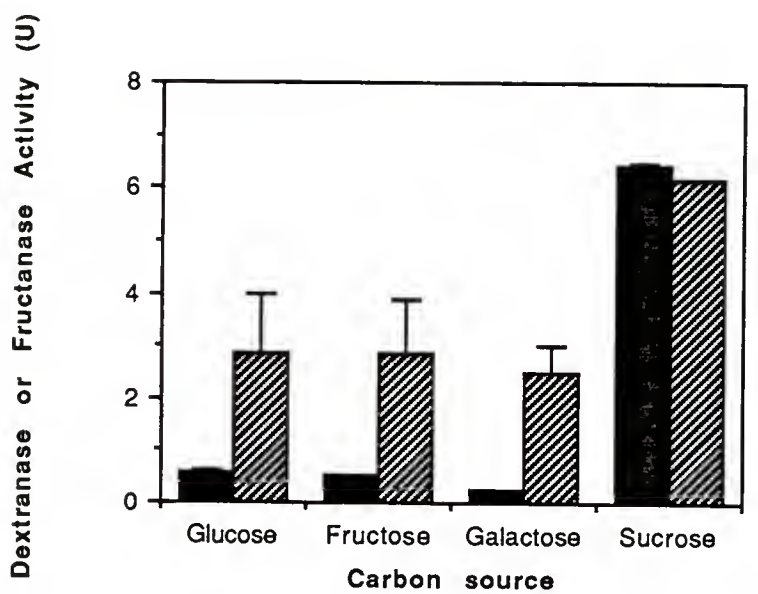


Figure 2-2. Effect of carbon source on dextranase and fructanase production by *S. salivarius* PC-1

Cells were grown in CDM/glucose (10 mM) and transferred to CDM containing either; glucose, fructose, galactose, or sucrose (10 mM each). When cultures reached Klett 75, supernatants were assayed for dextranase (solid bars) and fructanase (hatched bars). One unit of enzyme activity (U) was defined as the amount of dextranase or fructanase catalyzing the release of 1 μmol reducing sugar (glucose or fructose) min^{-1} ml^{-1}. Each bar represents the mean ± SD of triplicates from a representative experiment.

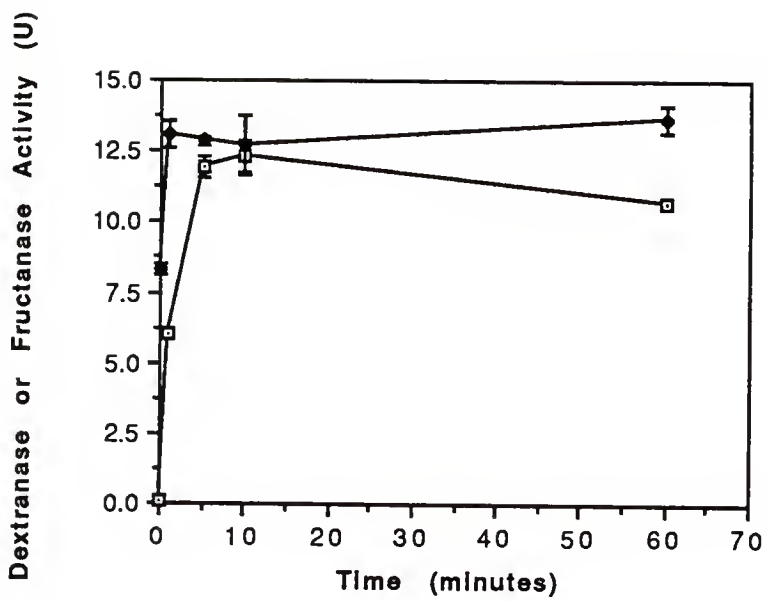


Figure 2-3. Effect of sucrose shift on production of dextranase and fructanase in *S. salivarius* PC-1

Sucrose (10 mM) was added to cells grown in CDM/galactose (10 mM) to Klett 75. Aliquots were removed at time intervals from suspensions held at 37°C. Supernatants were assayed for dextranase (open squares) and fructanase (closed squares) activities. One unit of enzyme activity (U) was defined as the amount of dextranase catalyzing the release of 1 μmol reducing sugar (glucose or fructose) min^{-1} ml^{-1}. Each data point shows the mean ± SD of triplicates from a representative experiment.

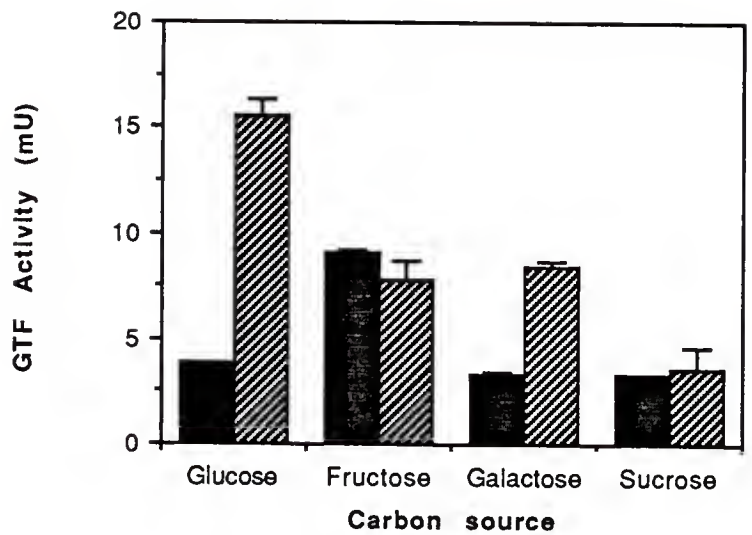


Figure 2-4. Effect of carbon source on cell-associated and extracellular GTF production in *S. salivarius* PC-1

Cells were grown in CDM/glucose (10 mM) and transferred to CDM containing either; glucose, fructose, galactose, or sucrose (10 mM each). Cultures were harvested at Klett 75 after growth at 37°C and cell-associated (solid bars) and extracellular (hatched bars) GTF activities were assayed. One unit of enzyme activity (U) was defined as the amount of GTF that catalyzed the incorporation of 1 nmol of the glucose moiety of labeled sucrose min^{-1} ml^{-1}. Each bar represents the mean $\pm$ SD of triplicates from a representative experiment.

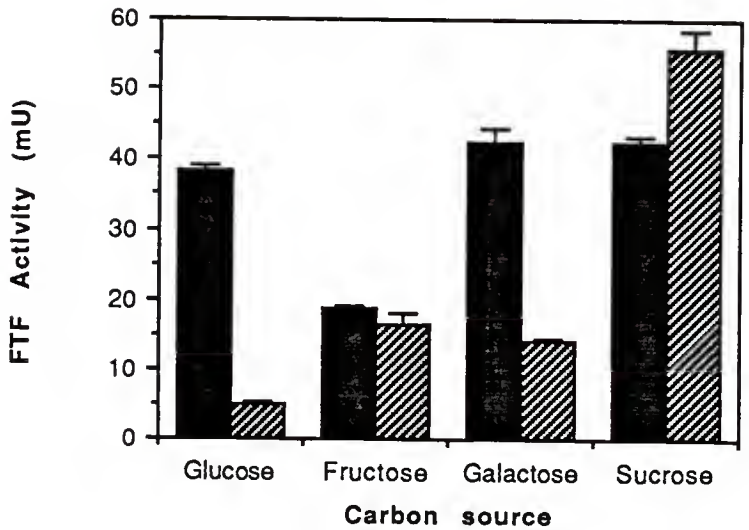
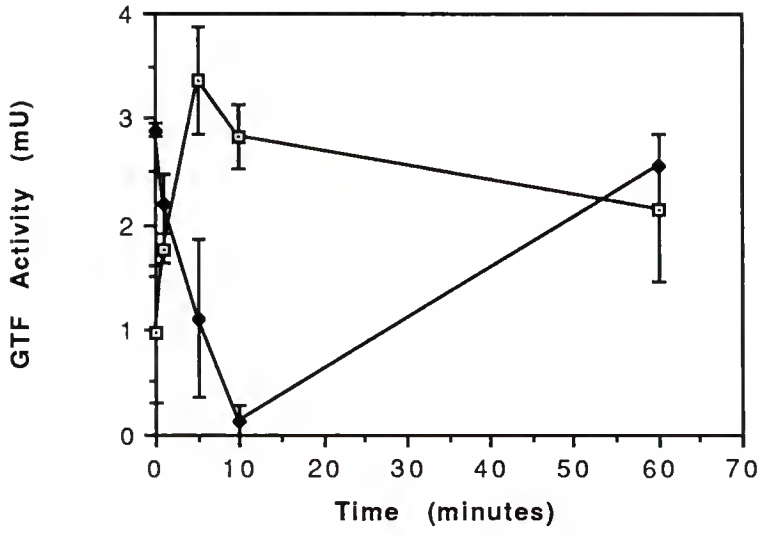


Figure 2-5. Effect of carbon source on cell-associated and extracellular FTF production in *S. salivarius* PC-1

Cells were grown in CDM/glucose (10 mM) and transferred to CDM containing either; glucose, fructose, galactose, or sucrose (10 mM each). Cultures were harvested at Klett 75 after growth at 37°C and cell-associated (solid bars) and extracellular (hatched bars) FTF activities were assayed. One unit of enzyme activity (U) was defined as the amount of FTF that catalyzed the incorporation of 1 nmol of the fructose moiety of labeled sucrose min^{-1} ml^{-1}. Each bar represents the mean $\pm$ SD of triplicates from a representative experiment.

a



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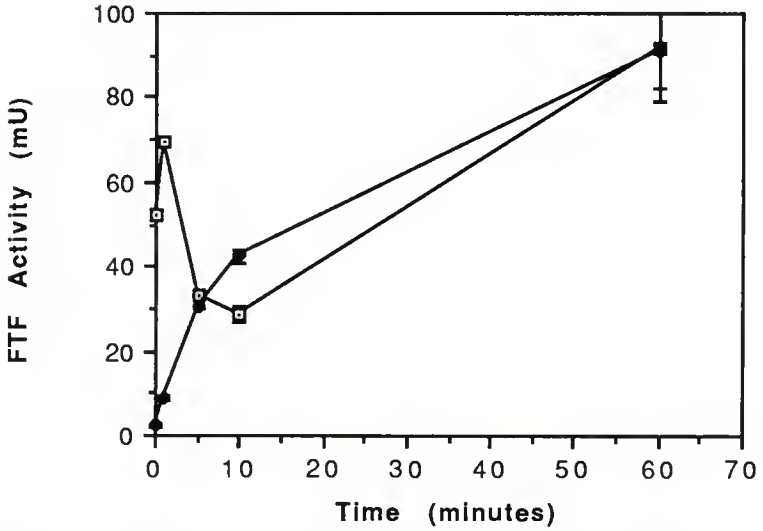


Figure 2-6. Effect of sucrose shift on production and distribution of GTF and FTF in *S. salivarius* PC-1.

Sucrose (10 mM) was added to *S. salivarius* PC-1 grown in CDM/galactose (10 mM) at 37°C to Klett 75. Aliquots were removed at timed intervals as shown and cell-associated (open squares) and extracellular (closed squares) glycosyltransferase activities were determined. (A) represents GTF activity, (B) represents FTF activity. One unit of enzyme activity (U) was defined as the amount of GTF or FTF that catalyzed the incorporation of 1 nmol of the glucose or fructose moiety, respectively, of labeled sucrose min^{-1} ml^{-1}. Each data point shows the mean $\pm$ SD of triplicates from a representative experiment.

Table 2-1. Effect of Antibiotics on Dextranase and Fructanase Production

Treatment	Enzyme Activity (U)	
	Dextranase	Fructanase
None	0.16 ± 0.03	6.58 ± 0
S†	10.67 ± 0	11.72 ± 0.47
C*	0.10 ± 0.01	6.27 ± 0.12
S&C	9.89 ± 0.64	9.68 ± 0.60
R‡	0.10 ± 0.03	6.98 ± 0.38
S&R	10.43 ± 0.25	8.76 ± 0.23

†S = Sucrose

*C = Chloramphenicol

‡R = Rifampicin

A *S. salivarius* PC-1 CDM/galactose culture was grown to mid-exponential phase (Klett 75). Sucrose (10 mM), chloramphenicol (100 mg ml^{-1}), rifampicin (100 mg ml^{-1}), or combinations of these were added to aliquots of the culture and then incubated at 37°C for 1 hour. Control cultures contained none of these reagents. The supernatants were assayed for extracellular dextranase and fructanase activities. One unit of enzyme activity (U) was defined as the amount of dextranase or fructanase catalyzing the release of 1 μmol reducing sugar min^{-1} ml^{-1}.

Table 2-2. Effect of Antibiotics on Glucosyltransferase Production

Treatment	GTF Activity (mU) Cell-Associated	Extracellular	Total
None	0.74 ± 0.13	2.95 ± 0.84	3.69
S†	3.52 ± 0.10	3.43 ± 0.12	6.95
C*	0.34 ± 0.09	2.86 ± 0.06	3.20
S&C	3.21 ± 0.04	1.08 ± 0.24	4.29
R‡	0.43 ± 0.03	5.44 ± 0.23	5.87
S&R	3.31 ± 0.07	1.00 ± 0.02	4.31

†S = Sucrose

*C = Chloramphenicol

‡R = Rifampicin

A *S. salivarius* PC-1 CDM/galactose culture was grown to mid-exponential phase (Klett 75). Sucrose (10 mM), chloramphenicol (100 mg ml^{-1}), rifampicin (100 mg ml^{-1}), or combinations of these were added to aliquots of the culture and then incubated at 37°C for 1 hour. Control cultures contained none of these reagents. The cells and supernatants were assayed for GTF activity. One unit of enzyme activity (U) was defined as the amount of GTF that catalyzed the incorporation of 1 nmol of the glucose moiety of labeled sucrose min^{-1} ml^{-1}.

Table 2-3. Effect of Antibiotics on Fructosyltransferase Production

Treatment	FTF Activity (mU) Cell-Associated	Extracellular	Total
None	31.44 ± 0.46	5.06 ± 0.01	36.50
S†	40.31 ± 1.76	34.38 ± 0.25	74.69
C*	35.76 ± 1.94	5.01 ± 0.01	40.77
S&C	11.52 ± 0.03	30.77 ± 0.15	42.29
R‡	47.28 ± 0.26	4.03 ± 0.09	51.31
S&R	10.89 ± 0.01	26.67 ± 1.91	37.56

†S = Sucrose

*C = Chloramphenicol

‡R = Rifampicin

A *S. salivarius* PC-1 CDM/galactose culture was grown to mid-exponential (Klett 75). Sucrose (10 mM), chloramphenicol (100 mg ml^{-1}), rifampicin (100 mg ml^{-1}), or combinations of these were added to aliquots of the culture and then incubated at 37°C for 1 hour. Control cultures contained none of these reagents. The supernatants were assayed for FTF activity. One unit of enzyme activity (U) was defined as the amount of FTF that catalyzed the incorporation of 1 nmol of the fructose moiety of labeled sucrose min^{-1} ml^{-1}.

CHAPTER 3
THE EXTRACELLULAR ENDODEXTRANASE OF *STREPTOCOCCUS SALIVARIUS*: MOLECULAR CLONING AND STUDIES OF ENZYME REGULATION

Introduction

Streptococcus salivarius, although not a major oral pathogen, is a common inhabitant of the oral cavity (Weerkamp & McBride, 1980). This species is among the earliest colonizers of the human mouth after birth and constitutes about 40-60% of the streptococci in saliva and on the tongue dorsum (Hamada & Slade, 1980). Its presence in large numbers, and therefore its potential impact on the oral ecology (Staat *et al.*, 1982), make the study of this organism and the extracellular enzymes it produces, e.g. glycosyltransferases, fructanase, and dextranase (Chassy *et al.*, 1976; Takahashi, *et al.*, 1983; Houck *et al.*, 1987) of considerable importance. Understanding the regulation and control of these enzymes which are involved in sucrose metabolism will be necessary for a better understanding of the role of this organism in the oral cavity.

Until recently, these enzymes had been assumed to be constitutive, as they are produced in the absence of substrate (Janda & Kuramitsu, 1978; Wenham *et al.*, 1979). However, it now has been shown that sucrose plays a role in the regulation of its metabolism in *S. salivarius* and that significant versatility is maintained in the mechanisms by which this is accomplished (Townsend-Lawman & Bleiweis, 1991). Preliminary work

has shown that this organism uses a variety of mechanisms to control this set of enzymes, allowing the regulation of their expression and function at several widely separated stages in their production. Among these, dextranase (α-1,6-glucan hydrolase, EC 3.2.1.11) appears to be the only enzyme controlled primarily at the posttranslational level. Dextranase activity increased immediately upon the addition of sucrose to galactose-grown cells, a phenomenon which was not affected by inhibitors of transcription (rifampicin) or translation (chloramphenicol). These results suggest that the increase in dextranase activity, in response to sucrose, may involve the displacement of a dextranase inhibitor (Townsend-Lawman & Bleiweis, 1990). This chapter describes experiments which further suggest the presence of such an inhibitor, its displacement by sucrose, and also indicate other levels of posttranslational control of this enzyme. Genetic and biochemical approaches have been taken in order to study the coordination of these regulatory events and the biological significance of dextranase to this organism.

Methods

Bacterial strains and bacteriophages.

Escherichia coli XL1-Blue: *endAI, hsdR17* (rk-, mk+), *supE44, thi-1, lambda-, recA1, gyrA96, relA1,* (*lac-*) [F', *proAB, lacI^{q}ZΔ M15, Tn10* (*tet^{R}*)] was purchased from Stratagene. XL1-Blue was maintained on Luria-Bertani (LB)/tetracycline (12.5 μg ml^{-1}) (Sigma Chemical Co.) agar plates and routinely grown in LB medium (Maniatis *et al.*, 1982). To prepare the XL1-Blue host cells for all manipulations described in this manuscript a single colony was inoculated into LB broth supplemented with

0.2% maltose and 10 mM $MgSO_4$. The cultures were grown overnight at 37°C with vigorous shaking, centrifuged at 1,000*g* for 10 minutes, and resuspended in 0.5 volumes of 10 mM $MgSO_4$. *Streptococcus salivarius* PC-1, a fresh isolate (Townsend-Lawman & Bleiweis,1991), was grown in chemically defined medium (CDM) (Terleckyj *et al.*, 1975) and stored at -70°C in 25% (v/v) glycerol. *Streptococcus salivarius* PC-1 CDM culture supernatants were prepared by removing the cells by centrifugation (4,000*g*, 20°C, 5 minutes) followed by filtration using a 0.2 μm membrane filter (Gelman Sciences, Inc.). The culture supernatants were concentrated using Centriprep™ concentrators (Amicon) and dialyzed overnight at 4°C in potassium phosphate buffer, pH 6.35.

Isolation of *S. salivarius* genomic DNA.

Chromosomal DNA was isolated from *S. salivarius* PC-1 as follows: PC-1 cells were grown in 200 ml CDM from an overnight inoculum in the same medium to an $O.D._{600}$ of 0.6. The culture was centrifuged for 10 minutes at 10,000*g*. Cells were washed once with ice-cold 2 M NaCl (100 ml) and once with ice-cold dH_2O (100 ml) and centrifuged for 10 minutes at 10,000*g*. The cell pellet was resuspended in 4.5 ml glucose-Tris (GT) buffer (20 mM Tris, pH 7.0, and 20% glucose) containing 0.5 mg mutanolysin (Sigma Chemical Co.) and incubated at 37°C for 1 hour. One ml sucrose-Tris-EDTA (STE) buffer (1% sucrose, 100 mM Tris, pH 8.0, and 200 mM EDTA, pH 8.0) was added and incubated at 37°C for 15 minutes. The reaction mixture was chilled briefly on ice and 1.5 ml 5 M sodium percholate was added. The lysate was extracted with 7.5 ml phenol/chloroform (1:1), shaken gently for 10 minutes, and centrifuged at

5,000*g* for 10 minutes. This extraction step was repeated 4 times. Genomic DNA was precipitated by mixing 2 volumes of ice-cold 95% ethanol with the aqueous solution, freezing at -70°C for 20 minutes and centrifuging at 10,000*g* for 10 minutes at 4°C. The DNA pellet was washed once with 70% ethanol, air dried, and dissolved in 1 ml Tris-EDTA (TE) buffer (10 mM Tris, pH 7.4 and 1 mM EDTA, pH 8.0). RNase (Sigma Chemical Co.) was added to a final concentration of 100 μg ml^{-1} and incubated at 37°C for 1 hour. Proteinase K (Sigma Chemical Co.) was added to a final concentration of 200 μg ml^{-1} and incubated at 37°C for 2 hours. This solution was extracted once with an equal volume of chloroform. The DNA was precipitated with ethanol and resuspended in 1 ml TE.

Construction of the *S. salivarius* genomic library.

The *S. salivarius* chromosomal DNA was partially digested with *Eco* R1 (Promega), and ligated to *Eco* R1-cut and dephosphorylated Lambda ZAP II vector arms (Stratagene) using T4 DNA ligase (IBI). After *in vitro* packaging using a packaging extract from Stratagene, the genomic library was generated by infecting XL1-Blue indicator cells. To test the quality of the packaged ligation product, samples (1-500 μl) of the packaged reaction were plated with 200 μl XL1-Blue cells. Phage and bacteria were preincubated at 37°C for 15 minutes. Three milliliters of NZ Amine (NZY) top agar (0.5% NaCl, 0.2% $MgSO_4.7\ H_2O$, 0.5 % yeast extract, and 1% casein hydrolysate) containing 50 μl of 0.5 M isopropyl-β-thio-galactopyranoside (IPTG, in H_2O) and 50 μl of 125 mg ml^{-1} 5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside (X-gal) in N,N-dimethylformamide (DMF) were added to phage and bacteria and plated onto NZY agar plates.

Screening the *S. salivarius* genomic library for dextranase clones.

In order to isolate dextranase clones, recombinant phage from the genomic library were infected into XL1-Blue indicator cells, mixed with 8 ml soft agar containing NZY medium and 1% blue dextran (Sigma Chemical Co.) to give approximately 1400 plaques per 150 mm petri dish and overlaid onto M9 agar (Maniatis *et al.*, 1982). Dextranase clones were identified as plaques surrounded by zones of clearing on the blue dextran agar (Barrett *et al.*, 1987) and were plaque purified for future use (Figure 3-1a). Recombinant phage expressing dextranase activity appeared at a frequency of 1.2×10^{-3}.

Preparation of *E. coli* lysates and *S. salivarius* supernatants expressing dextranase activity.

Dex phage lysates were prepared as follows: Dextranase-positive plaques were cored from the blue dextran agar plate and transferred to a sterile microfuge tube containing 500 µl SM (0.58% NaCl, 0.2% $MgSO_4$, 50 mM Tris, pH 7.5, and 0.01% gelatin) and 20 µl chloroform. One milliliter of this phage stock was infected into 5 ml XL1-Blue cells and incubated at 37°C for 15 minutes. LB medium (1 L) containing 0.2% maltose and 10 mM $MgSO_4$ was added and the culture incubated at 37°C for 12 hours with vigorous shaking. Aliquots of these lysates and in separate experiments, PC-1 CDM culture supernatants, were precipitated with ammonium sulfate. The bacterial debris was pelleted (8,000*g*, 45 minutes, 4°C) and the supernatant filtered with 47 mm metrigard superfine prefilters (Gelman Instrument Co.) and sterilized with 1000 ml Nalgene™ sterilization filter units, Type S, CN (Nalge Co.). Sodium azide, to 0.02%,

was added along with 430 g L^{-1} solid ammonium sulfate (Fisher Scientific Co.). These solutions were stirred at 4°C for 72 hours. The resulting precipitate was collected by centrifugation (9,000*g*, 4°C, 4 hours), resuspended in a small volume of potassium phosphate buffer, pH 6.35, dialyzed overnight with frequent changes of the same buffer, and stored at 4°C until use. The phage lysates and supernatants were monitored periodically for dextranase activity by radial diffusion from wells cut in a 1% agarose gel matrix containing 0.2% blue dextran and 50 mM potassium phosphate buffer, pH 6.35. Activity was visualized as zones of clearing surrounding the wells.

Protein analysis of native and recombinant dextranases.

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 7.5% gels by using the discontinuous buffer system of Laemmli, (1970). High range prestained SDS-PAGE standards (Bio-Rad Laboratories) were run simultaneously in order to determine approximate molecular weight(s) of the PC-1 and recombinant proteins. SDS has been shown to inhibit dextranase activity (Barrett & Curtiss, 1986). To circumvent this problem, the proteins from PC-1 CDM culture supernatant (Centriprep-concentrated and ammonium sulfate-precipitated) and *dex* phage lysates (unconcentrated and ammonium sulfate-precipitated) were transferred onto nitrocellulose membranes at 200 mA for 1 hour in the buffer of Towbin *et al.*, (1979). The blots were laid over a 1% agarose gel matrix containing 0.4% blue dextran and 50 mM potassium phosphate buffer, pH 6.35, and incubated at 37°C overnight. The proteins were able to renature sufficiently such that dextranase activity

was visualized as clear bands surrounded by blue color caused by the infiltration of blue dextran from the gel to the nitrocellulose filter.

Collection of polysaccharides from *S. salivarius* recombinants.

Recombinant clones encoding sucrolytic enzymes were selected from the *S. salivarius* genomic library by their ability to produce polysaccharide on M9 agar containing 1% sucrose (Gilpin *et al.*, 1985). Since *E. coli* XL1-Blue cannot utilize sucrose as a carbon source, NZY soft agar was used to give a weak bacterial lawn. Plaques surrounded by substantial growth suggested the presence of cloned *S. salivarius* sucrolytic enzymes. These plaques appeared at a frequency of 3.3×10^{-3}. One third of these plaques produced globules of polysaccharide when incubated for longer periods of time (Figure 3-1b). Ten such plaques (recombinants PG1 to PG10) were plaque-purified and replated on XL1-Blue. The plates were incubated at room temperature for 2 weeks. The polysaccharide was scraped from the surface of the agar and dissolved in water (20 ml). After pelleting the agar and cell debris by centrifugation (4,000*g*, 20°C, 5 minutes), two volumes of ice-cold 95% ethanol were added to precipitate the polysaccharide, which was spooled out with a capillary tube. The polysaccharide was resuspended in water (20 ml) and extracted with ethanol at least twice before being air-dried and weighed.

Determination of the sugar component in purified polysaccharides.

In order to determine whether the gene encoding glucosyltransferase (*gtf*) or fructosyltransferase (*ftf*) had been cloned and which type of polymer (glucan or fructan) was being produced, the sugar component(s)

of the polysaccharides was determined by thin layer chromatography (TLC) following acid hydrolysis. Polysaccharides from the recombinants (20 mg), levan (20 mg), and dextran Type 100C (20 mg), were boiled (100°C, 1 hour) under N_2 in sealed glass ampules with 2 N HCl. Two microliters samples of these reactions, along with 2 µl (fructose, sucrose, and glucose) (1 M) as standards, were loaded onto a dry silica gel plate which had been equilibrated overnight with the separation solvent (chloroform-acetic acid-water, 3.0: 3.5: 0.5, v/v). After separation in the solvent, the sugars were visualized by spraying the plate with 10 ml of 1% diphenylamine (wt/v), 1% analine (v/v), acetone mixed with 1 ml 85% phosphoric acid, and heating (130°C, 10 minutes) (DeStefanis & Ponte, 1968). R_f values of each spot were calculated as a ratio of the distance between the origin and the center of the spot to the distance between the origin and the solvent front. Crude recombinant lysates also were assayed for GTF and FTF activity by incorporation of radioactivity from differentially labeled sucrose (Robrish *et al.*, 1972).

Determination of dextranase activity.

Dextranase activity was determined using a standard assay for reducing sugars designed by Somogyi (1951) and Nelson (1944). Dextran Type 100C (Sigma Chemical Co.) was incubated with the enzyme-containing preparation at a final concentration of 0.25 mg ml^{-1}, unless specified otherwise. The reactions were stopped after 180 minutes. The amount of reducing sugar released was determined by comparison with glucose standards. One unit of enzyme activity (U) was defined as the amount of dextranase catalyzing the release of 1 µmol glucose min^{-1} ml^{-1}.

Each data point represents the mean (+/- SD) of triplicates from a respresentative experiment.

Carbon source utilization by *S. salivarius* PC-1.

Growth curves were generated by inoculating triplicates of CDM or CDM/substrate with a CDM/glucose (10 mM) starter culture at a 1:10 ratio. Substrates employed as carbon sources included glucose, sucrose, dextran Type 100C, dextran Type 500C, dextran Type 2000C, isomaltose (IM_2), isomaltotriose (IM_3), isomaltotetraose (IM_4), isomaltopentaose (IM_5) (Sigma Chemical Co.), and glucan obtained from *S. salivarius gtf* recombinant PG10, at final concentrations of 1 mg ml^{-1}. Optical density measurements were taken with a Klett-Summerson photoelectric colorimeter (Filter No. 54) every 30 minutes until stationary phase was reached. The pH of each culture was determined at the same time intervals using ColorpHast™ indicator sticks (MC/B Manufacturing Chemists, Inc.).

Substrate specificity of native and recombinant dextranases and product analysis.

Tenfold concentrated *S. salivarius* PC-1 CDM/sucrose (10 mM) culture supernatant and ammonium sulfate precipitated *dex* phage PD1 lysate were incubated with blue dextran, dextran Type 100C, dextran Type 500C, dextran Type 2000C, or glucan obtained from *S. salivarius gtf* recombinant PG10 at final concentrations of 0.25 mg ml^{-1}, for 180 minutes. Dextranase activity was determined as above. Duplicate reactions were incubated overnight and analyzed by TLC, using the same solvent system as described previously, to determine the products of native and

recombinant dextranase activity. Standards used were glucose and the oligosaccharides of the isomaltose series (IM_2, IM_3, IM_4, IM_5).

Sucrose-mediated release of dextranase inhibition in PC-1 cell-free supernatant.

To compare the levels of dextranase activity in mid-exponential *S. salivarius* PC-1 cultures grown with or without sucrose, tenfold concentrated PC-1 culture supernatants (CDM-galactose or sucrose, 10 mM) were incubated with dextran Type 100C, at a final concentration of 0.25 mg ml^{-1} and assayed for dextranase activity at 15 minute intervals. To confirm that the increase in dextranase activity in response to sucrose occurred after dextranase entered the extracellular environment, sucrose at several concentrations (0-14 mM), was added to sterile, concentrated culture supernatant from *S. salivarius* PC-1 cells grown in CDM with 10 mM galactose and incubated at 37°C for 1 hour. Dextranase activity in this supernatant was quantitated by measuring the amount of reducing sugar released from dextran Type 100C.

Effects of untreated and sucrose-treated cell-free *S. salivarius* supernatants on recombinant dextranase activity.

Dialyzed, concentrated PC-1 CDM/galactose (10 mM) culture supernatant was prepared as above. Sucrose (10 mM) was added to an aliquot of the CDM/galactose supernatant and incubated at 37°C for 1 hour. These supernatants, CDM/galactose and CDM/galactose plus sucrose, were added in 0, 10, 20, 30, 40, and 50% proportions to a constant amount (25 µl) of ammonium sulfate precipitated recombinant *dex* lysate (PD1). Controls with 100% supernatant and no recombinant enzyme were

included to provide estimates of background activity. Dextran Type 100C was added to a final concentration of 0.25 mg ml^{-1} and incubated for 180 minutes at 37°C. Dextranase activity was determined by measuring the amount of reducing sugar released. Substrate concentration and enzyme activity (U) were such that the limiting component was substrate. Therefore, whether the entire reaction mixture (50 µl) consisted of the enzyme preparation (*S. salivarius* supernatant or PD1 lysate) or half the reaction mixture (25 µl enzyme preparation, 25 µl 50 mM potassium phosphate buffer, pH 6.35), measurable dextranase activity was the same due to limiting dextran.

Results

Construction and screening of the *S. salivarius* genomic library.

Streptococcus salivarius DNA was cloned into Lambda ZAP II after partial digestion with the restriction endonuclease, *Eco* R1, and ligation with T4 DNA ligase. The phage library was used to infect *E. coli* XL1-Blue which was plated on M9 agar plates containing sucrose. Clones expressing sucrase activity appeared as rings of *E. coli* growth surrounding individual plaques and occurred at a frequency of 3.3×10^{-3}. Upon further incubation, 33% of these sucrolytic clones were able to form domes of polysaccharide from the sucrose (Figure 3-1a). Ten polysaccharide-positive clones (PG1 to PG10) expressed GTF, but not FTF, activity when assayed with differentially labeled sucrose in the standard glycosyltransferase assay (Lawman & Bleiweis, unpublished results). In addition, the polysaccharides produced by these clones were hydrolyzed with 2N HCl and analyzed by TLC, and found to contain only glucose

(Lawman & Bleiweis, unpublished results). The glucan from PG10 was used later in this study to test the substrate specificity of native and recombinant dextranases. The *S. salivarius* library transduced into XL1-Blue also was plated on blue dextran (Figure 3-1b). Clones able to clear zones by hydrolyzing the blue dextran appeared at a similar frequency as GTF-expressing clones (1.2×10^{-3}). One clone expressing dextranase activity, PD1, was chosen for further investigation and was used to characterize the *dex* gene and its product. PD1 was found to carry a 6.3 kb *Eco* R1 fragment encoding the *dex* gene and a promoter, as the clone was capable of expressing dextranase activity in the absence of IPTG. A more complete characterization of the *dex* gene is the subject of Chapter 4. This chapter focuses on the characteristics of the *dex* gene product.

Three polypeptides of molecular weights 190, 90, and 70 KD in unconcentrated PD1 lysates were shown to be responsible for the dextranase activity of PD1 by SDS-PAGE/electroblot, and blue dextran overlays (Figure 3-2, lane 3). Upon precipitation with ammonium sulfate, only the two smaller molecular weight species could be visualized. This may be the result of proteolysis during the concentration process (Figure 3-2, lane 4). The native dextranase had an apparent molecular weight of 110 KD by this procedure regardless of the method of concentration (Figure 3-2, lanes 1 and 2). This assay was not sensitive enough to detect dextranase activity in unconcentrated *S. salivarius* supernatants.

Carbon source utilization.

It was of interest to determine whether *S. salivarius* could metabolize either large molecular weight dextrans or the products of endodextranase hydrolysis, oligoisomaltosaccharides, since it has been postulated that

stored dextran might serve as an energy reservoir for this and other organisms (Parker & Creamer, 1971; Hamada *et al.*, 1975; Schachtele *et al.*,1975b; Ellis & Miller, 1977). *Streptococcus salivarius* PC-1 was unable to utilize any of the isomaltosaccharides or dextrans as measured by growth or shifts in pH (Table 3-1). In fact, PG10 glucan appeared to inhibit *S. salivarius* growth in sucrose as evidenced by the longer generation time (from 57 to 69 minutes) and the lower absorbance at maximal growth (125 to 75 Klett units) when glucan was added to sucrose at 1 mg ml^{-1}. Melibiose also was tested as a growth substrate. If a melibiose operon were present in *S. salivarius* as in *Streptococcus mutans* (Tao *et al.*, 1990), melibiose might induce the uptake of oligosaccharides and their utilization as a fermentable carbon source. Melibiose did not serve as a carbon source for *S. salivarius* PC-1, nor did it facilitate the utilization of dextran or isomaltosaccharides. However, it did extend the lag-time of cells grown in sucrose and in sucrose/PG10 glucan from 90 to 150 minutes and the lag-time of cells grown in glucose from 90 to 240 minutes, and decreased the density at which growth peaked (Table 3-1).

Substrate specificity of native and recombinant dextranases and product analysis.

Dextranase is thought to hydrolyze accumulated dextrans in dental plaque and release hexose for consumption by oral microorganisms. Therefore, it was important to determine the extent to which various dextrans might serve as substrates for this enzyme and the products of such reactions. It also was of interest to compare the substrate specificity and resultant reaction products of the native and recombinant dextranases. Tenfold concentrated and dialyzed *S. salivarius* CDM/sucrose culture

supernatant and ammonium sulfate-precipitated PD1 lysate were incubated with dextrans from various sources and of different molecular weights (*S. salivarius* was grown in CDM/sucrose so that the native dextranase would be in its active form). All dextrans tested served as substrates for both the native and recombinant enzymes, but to varying degrees (Figure 3-3). The native dextranase in CDM/sucrose culture supernatant released reducing sugars from the native PG10 glucan to greater extents, ranging from 18 to 41% than from the other substrates (Figure 3-3a). The recombinant enzyme, however, was far less active on the native glucan (50%) than was the native dextranase (Figure 3-3b), although the *dex* gene product showed equivalent or greater degrees of activity on the commercial dextrans (Figure 3-3b). Relative dextranase specific activities (native: recombinant) were as follows: PG10, 2.22; Blue dextran, 0.99; T100C, 0.52; T500C, 0.38; T2000C, 0.75)

TLC analysis of the reaction products of dextran degradation revealed that the native form of dextranase was not able to hydrolyze any of the glucan substrates to the smaller isomaltosaccharides released by the recombinant enzyme (Figure 3-4). Because TLC was unable to resolve the oligosaccharides produced by the native enzyme, it is presumed these products were greater than 7 glucosyl residues in length. Neither form of dextranase released free glucose.

Sucrose-mediated release of dextranase inhibition in *S. salivarius* PC-1 cell-free supernatants.

In order to demonstrate further the disparity of dextranase activity between *S. salivarius* grown in CDM/galactose vs. CDM/sucrose (Townsend-Lawman & Bleiweis, 1991), supernatants from mid-exponential

cultures were concentrated, dialyzed, and assayed for dextranase activity at 15 minute intervals. When *S. salivarius* was grown in CDM with galactose, dextranase activity was minimal (Figure 3-5a), however, when cells were grown in the presence of sucrose, the dextranase was active (Figure 3-5a), even after the putative "activator" (sucrose) had been removed by extensive dialysis. Maximal activity required 180 minutes incubation with substrate, although significant activity was measurable earlier. To confirm that sucrose-dependent increases in dextranase activity were not cell-mediated, sucrose, at several concentrations was added to sterile, concentrated *S. salivarius* CDM/galactose culture supernatant, incubated for 1 hour, and assayed for dextranase activity. Sucrose was able to "activate" the otherwise inactive dextranase in a concentration-dependent manner, with an optimum at 10 mM disaccharide (Compare Figure 3-5b with 3-5a). This provided further evidence that the sucrose effect was initiated postgenetically.

Effects of untreated and sucrose-treated cell-free *S. salivarius* supernatants on recombinant dextranase activity.

To determine whether the increase in dextranase activity upon the addition of sucrose was due to activation or release from inhibition, cell-free *S. salivarius* CDM/galactose supernatant, or CDM/galactose supernatant plus sucrose was added, at various concentrations, to PD1 lysate. CDM/galactose supernatant inhibited the recombinant dextranase activity in a concentration-dependent manner (Figure 3-6a). This confirmed the presence of a dextranase inhibitor in the CDM/galactose supernatant and implied that it was present in excess of the native dextranase, since 37% was the maximum inhibition obtained using 50%

CDM/galactose supernatant. On the other hand, the dextranase activity in CDM/galactose supernatant which had been treated with 10 mM sucrose was additive to recombinant dextranase activity (Figure 3-6b), suggesting that both native and recombinant forms of the enzyme were able to utilize the same substrate molecules.

Discussion

All indications are that dextranase has the ability to participate in dental plaque formation and modification through its effects (degradation and/or synthesis) (Staat & Schachtele, 1974; Hamada *et al.*, 1975) on the capsular polysaccharides, which are thought to be important in bacterial aggregation and adherence (Gibbons & Van Houte, 1975; Schachtele *et al.*, 1975a). However, the biological significance of this enzyme and its role(s) in sucrose metabolism have not been firmly established. Dextranase may act directly in conjunction with GTFs in the synthesis and modification of dextran polymers, by providing primer and/or branchpoints, and thus may be involved in determining the degree of dextran solubility (Walker, 1972; Germaine *et al.*, 1977), a property shown to be an important factor in colonization, plaque formation and caries development (Walker & Jacques, 1987). Alternatively, dextranase may break down the accumulated dextran in plaque, destabilizing the plaque matrix, and at the same time provide fermentable hexose for consumption (Parker & Creamer, 1971; Hamada *et al.*, 1975; Schachtele *et al.*, 1975b Ellis & Miller, 1977) and acid production (Wood, 1969) by plaque organisms. In this capacity extracellular endodextranase may act at the beginning of a catabolic pathway in a synergetic manner with an intracellular exodextranase, as

found in *S. mutans* (Burne *et al.*, 1986; Russell & Ferretti, 1990), to hydrolyze short term dextran reserves rapidly to glucose (Dewar & Walker, 1975). Studies were initiated, therefore, to determine the major function of endodextranase, either anabolic or catabolic, and its contribution to the physiology of *S. salivarius* and potential contribution to other inhabitants of the oral cavity.

Most information to date on dextranase in oral streptococci has been obtained from *Streptococcus sobrinus* (Germaine & Schachtele, 1976; Ellis & Miller, 1977; Walker *et al.*, 1981; Barrett *et al.*, 1987). Purification of this enzyme has proven difficult due to multiple molecular weight forms attributable to enzyme aggregation and/or protease modification. Barrett *et al.* (1987) developed a purification scheme for dextranase from *S. sobrinus* culture supernatant fluids in which the majority of dextranase was recovered in two forms, molecular weights 175 KD and 160 KD. Lower molecular weight forms, ranging from 160 KD to 125 KD, were thought to be proteolytic breakdown products of the 175 KD dextranase. When these investigators screened *S. sobrinus* gene libraries for dextranase activity, three phenotypes of recombinant clones were identified. The authors believe that it was unlikely that the variation in molecular weight was due to the cloning of two separate genes since genetic characterizations of *S. sobrinus* dextranase mutants suggested a single *dex* gene.

Reported in this chapter is the cloning of the endodextranase from *S. salivarius* PC-1. Dextranase-positive clones were recovered at a frequency of 1.2×10^{-3}. One such clone, designated PD1, was chosen for further study and was found to carry a 6.3 kb *Eco* RI fragment encoding the *dex* gene. It was deduced that this insert contained a promoter since the clone

was capable of expressing dextranase activity in the absence of IPTG, and enough of the *dex* structural gene to produce a protein with dextranase activity of at least 190 KD (see Figure 3-2). This protein was degraded into lower molecular weight species (90 and 70 KD) which retained the ability to hydrolyze blue dextran as did the *S. sobrinus* dextranase (Barrett *et al.*, 1987). The native dextranase; however, was recovered as a single 110 KD polypeptide. The fact that the native protein appeared to be smaller than the recombinant, may be due to proteolytic degradation by streptococcal enzymes or may represent a monomeric form of this enzyme. At this point the possibility that the recombinant enzyme may be a fusion protein cannot be ruled out.

Preliminary results of studies on the regulation of sucrose metabolism in *S. salivarius* were presented above. In these studies it was shown that extracellular dextranase activity was higher in sucrose-grown *S. salivarius* PC-1 cells compared to cells grown in glucose, fructose, or galactose; that dextranase activity increased 100-fold when sucrose was added to cells growing in galactose; and that this immediate increase was not affected by transcriptional or translational inhibitors. Ellis and Miller (1977) found a similar sucrose effect on dextranase activity in *S. mutans* 6715 (*S. sobrinus*) supernatants. These results suggested that the increase in the activity of native dextranase in response to sucrose may involve the displacement of a dextranase inhibitor and implied that *de novo* synthesis was not required for the production of dextranase.

Hamelik and McCabe (1982) concluded that the presence of an inhibitor in batch-grown culture fluids of *S. mutans* accounted for the absence of endodextranase activity in strains known to produce this

enzyme. To investigate further the regulation of dextranase by sucrose and the presence of a dextranase inhibitor in *S. salivarius*, CDM/galactose and CDM/sucrose culture fluids were assayed for their ability to release reducing sugars from dextran. The CDM/galactose supernatant showed little dextranase activity while the CDM/sucrose supernatant showed increasing activity over time (see Figure 3-5a). "Activation" was a posttranslational effect since dextranase activity increased in a concentration-dependent manner when sucrose was added to cell-free *S. salivarius* CDM/galactose supernatant (see Figure 3-5b).

It is clear from Figure 3-2 that native dextranase was active in the absence of sucrose when separated from other proteins in *S. salivarius* supernatants. Also, the recombinant dextranase was active in the absence of sucrose (Figures 3-1, 3-2), which argues against the need for an activator. To distinguish between activation and relief of inhibition, CDM/galactose supernatant was added in increasing concentrations to recombinant dextranase preparations. Figure 3-6a demonstrates the presence of a factor in *S. salivarius* CDM/galactose supernatant which inhibited recombinant dextranase activity. This inhibitor apparently was in excess of a 1:1 molar ratio to the native dextranase, since it was available for inhibition of the recombinant enzyme. If sucrose acts to relieve this inhibition, presumably by replacing dextranase as the ligand for the dextranse inhibitor, one would expect sucrose-treated supernatants to be non-inhibitory to the recombinant dextranase. Not only was the effect non-inhibitory (see Figure 3-6b), there was a synergetic effect, i.e., dextranase activity, in the presence of a constant amount of substrate, increased to twice that of either native or recombinant alone. This implied that both

forms of dextranase (native and recombinant) were able to release reducing sugars from the same substrate molecules. It was not clear; however, if the two enzymes released similar or different end-products when assayed with dextran substrates.

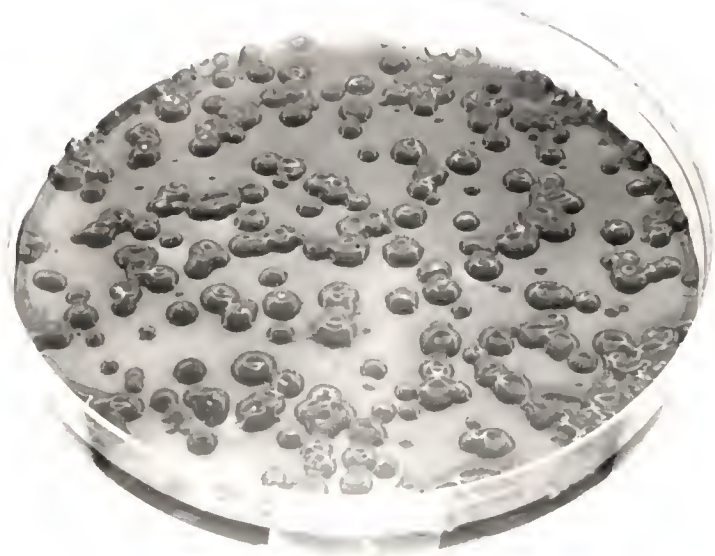
To compare enzyme activities, *S. salivarius* CDM/sucrose culture supernatant and ammonium sulfate-precipitated PD1 lysate were incubated with various dextrans. The native and recombinant enzymes were able to recognize all the dextrans tested, but varied in their recognition and/or hydrolysis of dextran substrates. The native dextranase hydrolyzed homologous glucan more successfully than dextrans from other sources, while the recombinant enzyme hydrolyzed heterologous (commercial) glucans to a greater degree than streptococcal capsular dextran (see Figure 3-3). The recombinant enzyme released the products expected from an endodextranase (from isomaltose to larger isomaltosaccharides, see Figure 3-4). On the other hand, the *S. salivarius* supernatant, which had been shown to release comparable levels of reducing activity (see Figure 3-3), did not hydrolyze any of the dextran substrates to small oligosaccharides or glucose (see Figure 3-4). It is thus likely that there was a factor present in the *S. salivarius* supernatant which regulated or directed the recognition, specificity and/or function of the native dextranase beyond its regulation by the dextranase inhibitor. Perhaps this "controlling" factor, possibly GTF itself, binds or otherwise modifies dextranase to limit and/or direct the degradation of the dextran to allow production of appropriately-sized primers or to pin-point insertion of branchpoints. Construction of isogenic *dex*$^-$ mutants would allow clarification of this vital functional activity.

Since *S. salivarius* PC-1 is unable to utilize dextrans, isomaltosaccharides, or melibiose as sole carbon sources (see Table 3-1), and since these substances may in fact inhibit growth, this organism probably does not utilize a synergetic pathway involving extracellular endodextranase, isomaltosaccharide transport factors, and intracellular exodextranase to metabolize stored capsular polysaccharides for energy. Ellis and Miller (1977) made similar conclusions from their work with *S. mutans* 6715. Induction or activation of dextranase by sucrose, but not dextran, would be an inefficient system if dextran was unable to serve as a fermentable carbohydrate. It also could be suggested that the melibiose transport operon found in *S. mutans*, enabling the utilization of melibiose and isomaltosaccharides may be missing in this strain of *S. salivarius*. Therefore, the major biological role of endodextranase in this organism appears to be to augment the activity of GTF(s) in a synthetic capacity.

It has been shown through genetic and biochemical means that the endodextranase of *S. salivarius* PC-1 acts as a component of the synthetic machinery designed to construct extracellular polysaccharides, and that its activity is directed by some factor present in the extracellular environment, perhaps GTF itself. This makes the positive regulation of dextranase activity by sucrose, the substrate for dextran formation by GTF(s), understandable. These findings suggest that the regulation of sucrose metabolism and the enzymes involved is more complex than once imagined. Implicit in these conclusions is the importance of immediate availability of critical enzymes on the cell's exterior to meet the extremely erratic signals from the environment (e.g. nutrient availability/deprivation). The extracellular availability of dextranase and certain glycosyltransferases may

be controlled to a great extent by posttranslational means rather than genetic regulation in this organism.

a



b

Figure 3-1. Screening the *S. salivarius* genomic library for *dex* and *gtf* recombinants.

(a) Recombinants from the *S. salivarius* library were selected for their ability to produce polysaccharide on M9 agar containing 1% sucrose. A plaque-purified *gtf* recombinant (PG10) exhibits the characteristic production of glucan from sucrose.

(b) Aliquots of the *S. salivarius* genomic library were mixed with NZY soft agar containing 1% blue dextran and overlaid onto M9 agar. Dextranase-positive (*dex*) clones were identified as plaques surrounded by a zone of clearing and plaque-purified for further study.

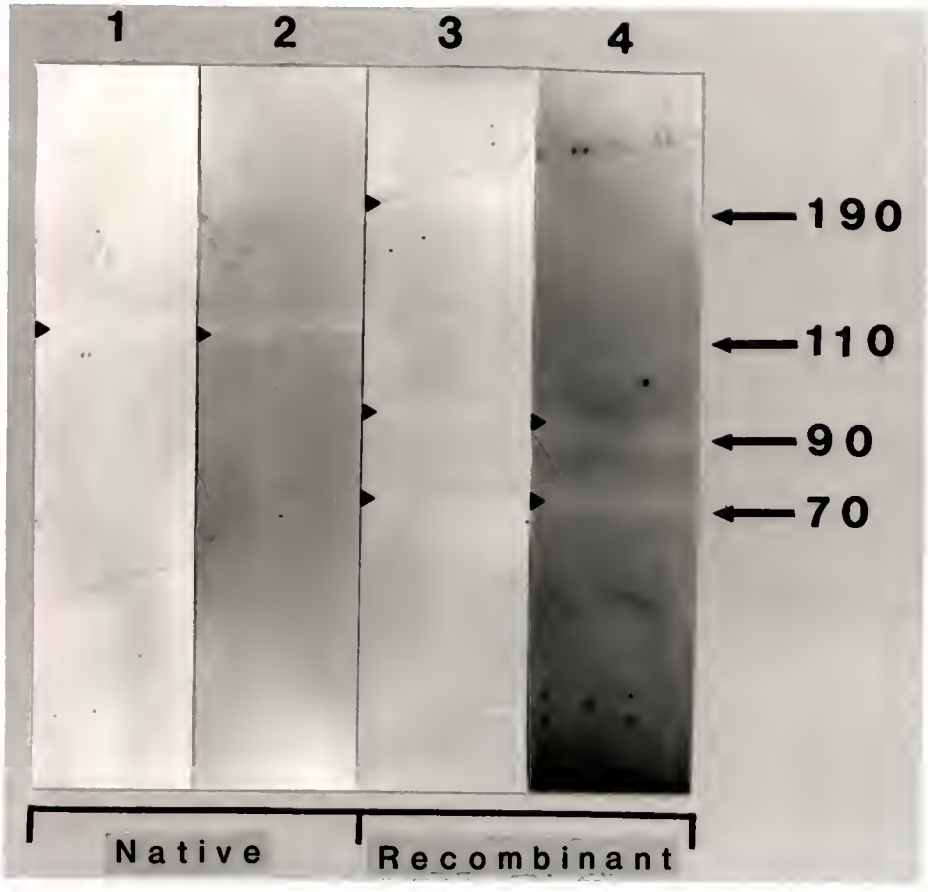
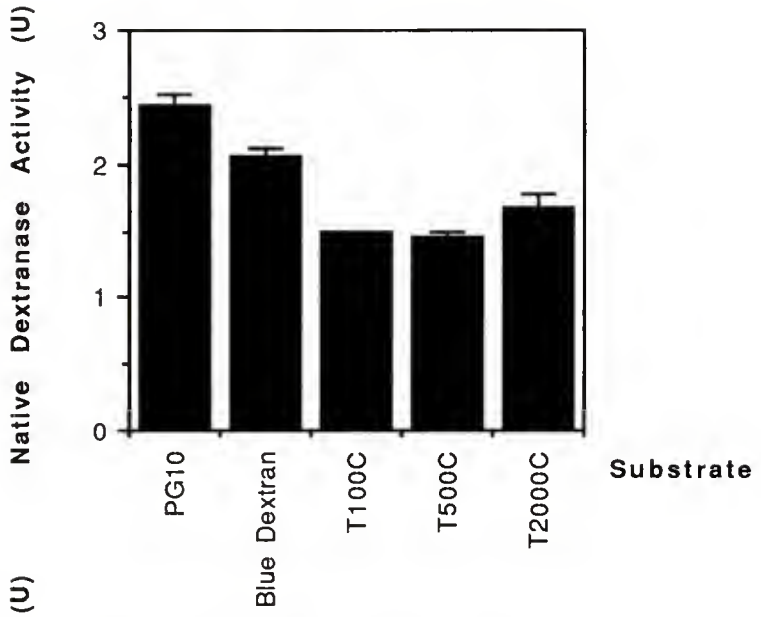


Figure 3-2. Detection of electroblotted dextranase activity on blue dextran-agarose.

Proteins were separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The blots were laid over a 1% agarose gel matrix containing 0.4% blue dextran and 50 mM potassium phosphate buffer, pH 6.35 and incubated overnight at 37°C. Dextranase activity was visualized as clear bands on the nitrocellulose membranes surrounded by blue color left by the infiltration of blue dextran from the gel. Lanes: 1) Centriprep-concentrated PC-1 CDM/sucrose culture supernatant, 2) ammonium sulfate-precipitated PC-1 CDM/sucrose culture supernatant, 3) unconcentrated *dex* phage lysate (PD1), 4) ammonium sulfate-precipitated *dex* phage lysate (PD1).

a



b

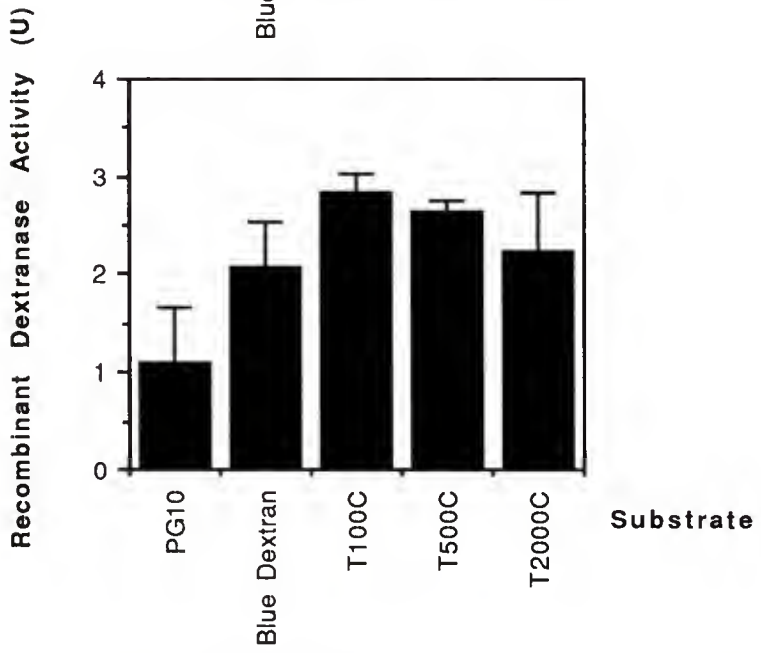


Figure 3-3. Substrate specificity of native and recombinant dextranases.

(a) Tenfold concentrated PC-1 CDM/sucrose (10 mM) culture supernatant and (b) ammonium sulfate-precipitated *dex* phage lysate were incubated with glucan obtained from *S. salivarius gtf* recombinant PG10, blue dextran, dextran Type 100C, dextran Type 500C, and dextran Type 2000C at final concentrations of 0.25 mg ml^{-1}. Dextranase activity was determined by the amount of reducing sugar released. One unit of enzyme activity (U) was defined as the amount of dextranase catalyzing the release of 1 μmol glucose min^{-1} ml^{-1}.

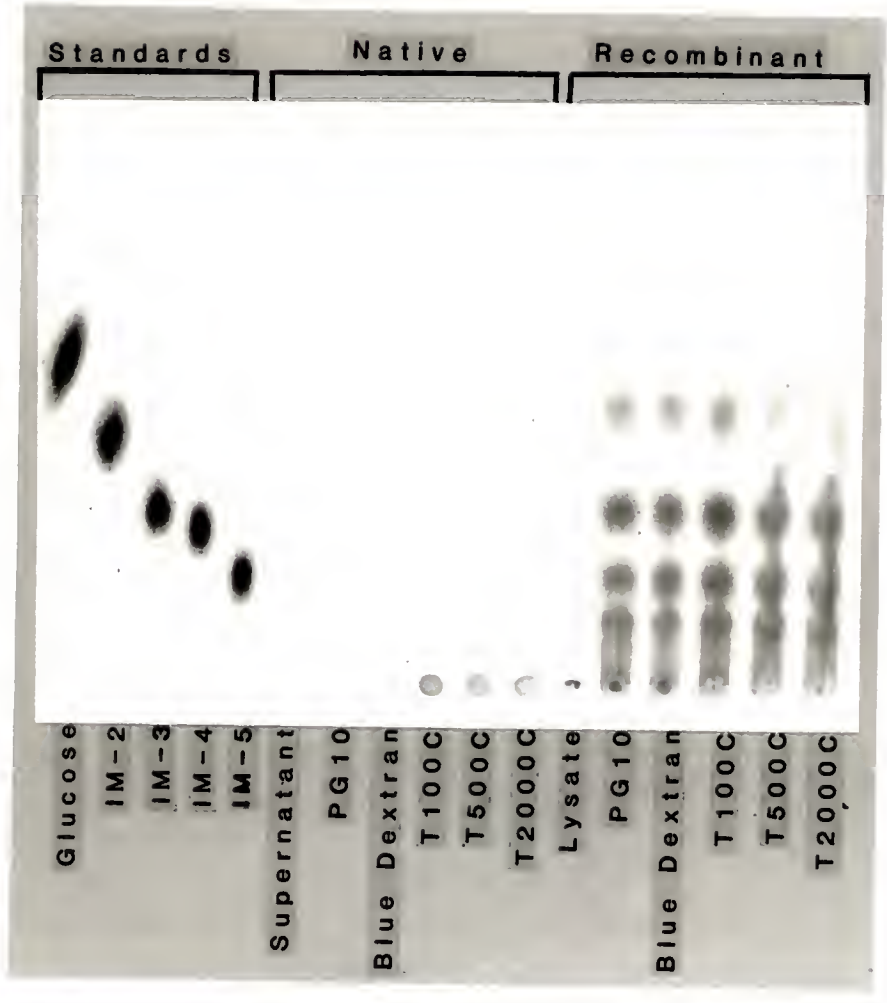
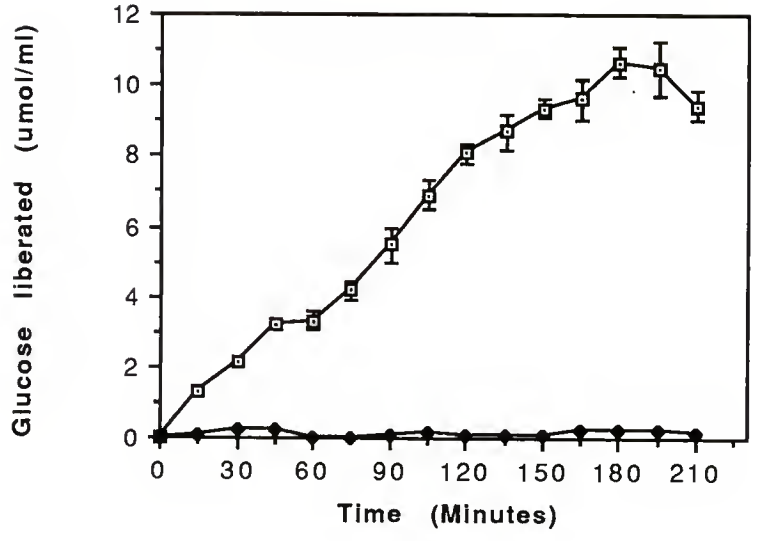


Figure 3-4. Product analysis of native and recombinant dextranases.

The products of the following reactions were separated by thin layer chromatography. Dialyzed, tenfold concentrated PC-1 CDM/sucrose (10 mM) culture supernatant (Native) and ammonium sulfate-precipitated PD1 *dex* phage lysate (Recombinant) were incubated with (1) no substrate, (2) glucan obtained from *S. salivarius* PC-1 *gtf* recombinant PG10, (3) blue dextran, (4) dextran Type 100C, (5) dextran Type 500C, and (6) dextran Type 2000C (at a final concentration of 10 mg ml^{-1}) overnight at 37°C. Standards include: G-glucose; IM_2-isomaltose; IM_3-isomaltotriose; IM_4-isomaltotetraose; IM_5-isomaltopentaose.

a



b

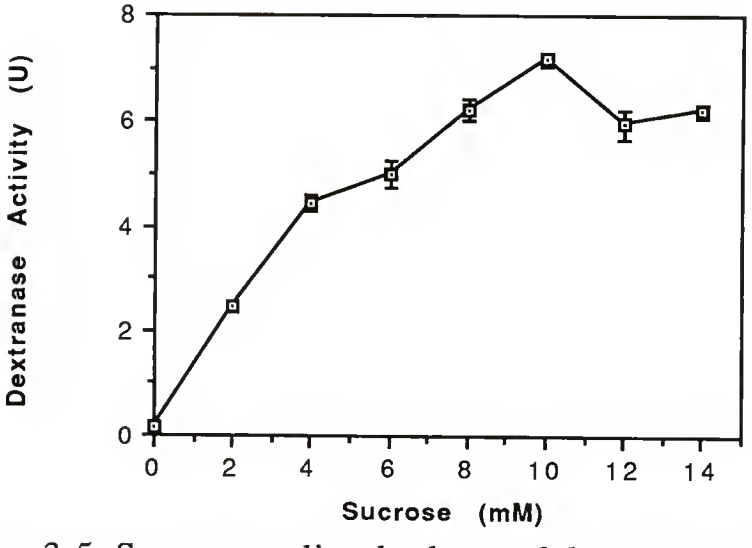
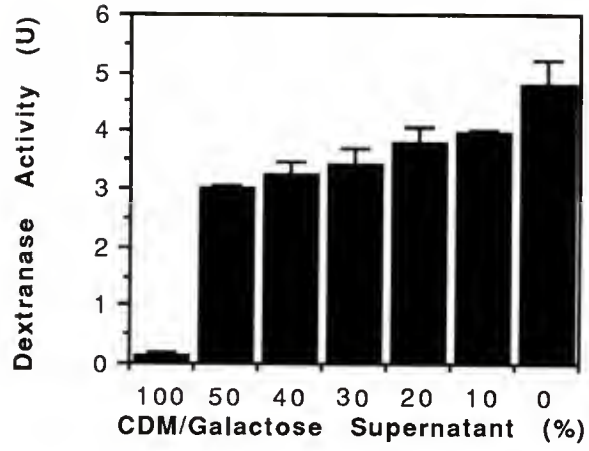


Figure 3-5. Sucrose-mediated release of dextranase inhibition in PC-1 cell-free supernatant.

(a) Tenfold concentrated culture supernatants from PC-1 grown in CDM with galactose (closed diamonds) or sucrose (open squares) were assayed for dextranase activity at 15 minute intervals. (b) Sucrose (0-14 mM) was added to sterile, concentrated culture supernatant from *S. salivarius* PC-1 cells grown in CDM with 10 mM galactose and incubated at 37°C for 1 hour. Dextranase activity was quantitated by measuring the amount of reducing sugar released from dextran Type 100C. One unit of enzyme activity (U) was defined as the amount of dextranase catalyzing the release of 1 μmol reducing sugar min^{-1} ml^{-1}. Assays were performed in triplicate.

a



b

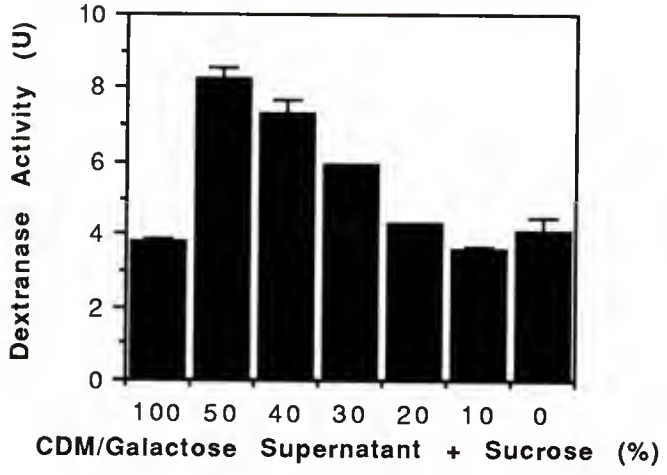


Figure 3-6. Effects of CDM/galactose and CDM/galactose plus sucrose cell-free *S. salivarius* PC-1 supernatants on recombinant dextranase activity.

(a) Tenfold concentrated PC-1 CDM/galactose (10 mM) culture supernatant and (b) tenfold concentrated PC-1 CDM/galactose (10 mM) culture supernatant incubated for 1 hour with sucrose (10 mM) were added in 0, 10, 20, 30, 40, and 50% proportions to ammonium sulfate-precipitated *dex* phage lysate. One hundred percent culture supernatants indicated the background levels of native enzyme in "inactive" (CDM/galactose) and "active" (CDM/galactose plus sucrose) supernatants. Dextran Type 100C was added to final a concentration of 0.25 mg ml^{-1} and incubated for 180 minutes at 37°C. Dextranase activity was determined by the amount of reducing sugar released. One unit of enzyme activity (U) was defined as the amount of dextranase catalyzing the release of 1 μmol glucose $min^{-1}ml^{-1}$.

Table 3-1. Carbon source utilization by *S. salivarius* PC-1.

Carbon source	Lag time (min)	Generation time (min)	Peak Absorbance (Klett units)	Final pH
Glucose	90	50	110	4
Sucrose	90	57	125	4
Melibiose	no growth	--	--	7
Melibiose + Glucose	240	72	85	4
Melibiose + Sucrose	150	54	75	4
§PG10	no growth	--	--	7
PG10 + Sucrose	90	69	75	4
PG10 + Melibiose	no growth	--	--	7
PG10 + Suc + Mel	150	54	65	4
*Dextrans or IMs (+/- Melibiose)	no growth	--	--	7

Streptococcus salivarius PC-1 cultures containing various carbon sources (1 mg ml^{-1}) or combinations thereof (1 mg ml^{-1} each) were monitored by Klett-Summerson colorimetric analysis for growth. Culture pH also was measured.

§ Glucan from the *S. salivarius gtf* recombinant PG10
* Blue dextran, dextran Type 100C, dextran Type 500C, dextran Type 2000C, IMs= isomaltosaccharides; IM_2-isomaltose; IM_3-isomaltotriose; IM_4-isomaltotetraose; IM_5 -isomaltopentaose

CHAPTER 4
ANALYSIS OF THE EXTRACELLULAR ENDODEXTRANASE GENE OF *STREPTOCOCCUS SALIVARIUS*

Introduction

Sucrose metabolism in the oral streptococci has been the subject of numerous studies, primarily because it is believed to be the major contributory factor in plaque formation and the subsequent development of dental caries (Drummer & Green, 1980; Hamada & Slade, 1980; Drucker *et al.*, 1984). The metabolism of sucrose, in its most basic form, involves the translocation of sucrose or its component hexoses (Slee & Tanzer, 1979), concomitant phosphorylation, hydrolysis, and utilization for energy (Saier, 1989). In a broad sense, however, it also includes the synthesis of extracellular polysaccharides, both glucans and fructans, catalyzed by glucosyltransferase (GTF) and fructosyltransferase (FTF) enzymes, respectively. These polymers are, in turn, degraded by dextranase (α-1,6-glucan hydrolase, EC 3.2.1.11), which has been shown to behave as an endoenzyme, breaking glucans down to isomaltosaccharides (Walker, 1972; Lawman & Bleiweis, submitted, Journal of General Microbiology, 1991), and fructanase (β-D-fructan fructohydrolase, EC 3.2.1.80), which hydrolyzes fructans to fructose (Burne *et al.*, 1987).

While sucrose metabolism has been the topic of much discussion as it relates to the potential virulence of oral streptococci, little attention has been focused on its potential to serve as a model for studying the complex

regulatory network of enzymes necessary for cellular response to irregular signals from the environment. *Streptococcus salivarius*, an unremarkable pathogen is; however, an adept colonizer of the human mouth and can constitute up to 60% of the streptococci in saliva and on the dorsal side of the tongue (Hamada & Slade, 1980). It possesses the entire array of enzymes, eg. glycosyltransferases, fructanase, and dextranase (Chassy *et al.*, 1976; Takahashi *et al.*, 1983; Houck *et al.*, 1987; Townsend-Lawman & Bleiweis, 1991), necessary for the production and degradation of the extracellular polysaccharides derived from sucrose and has been shown to regulate these enzymes in an extremely complex fashion (Townsend-Lawman & Bleiweis, 1991; Lawman & Bleiweis, submitted, Journal of General Microbiology, 1991). For these reasons, *S. salivarius* is the ideal species on which to base a model for the control of extracelluar enzymes in Gram positive organisms. Evidence that the availability of dextranase and certain glycosyltransferases is controlled to a great extent by posttranslational, rather than genetic regulation (Townsend-Lawman & Bleiweis, 1991; Lawman & Bleiweis, submitted, Journal of General Microbiology, 1991), lays the foundation for further studies and calls for indepth genetic studies of each enzyme involved.

The present chapter focuses on the analysis of the endodextranase of *S. salivarius* at a molecular level and includes the confirmation of the cloned dextranase activity, its molecular weight, the uniqueness of this sequence in the *S. salivarius* genome and a molecular map of the DNA which encodes it.

Methods

Bacterial strains and growth media.

Streptococcus salivarius PC-1, a fresh isolate (Townsend-Lawman & Bleiweis, 1991) was grown in chemically defined medium (CDM) (Terleckyj *et al.*, 1975) and stored at -70° in 25% (v/v) glycerol. A genomic library was constructed as described previously (Lawman & Bleiweis, submitted, Journal of General Microbiology, 1991). Briefly, *S. salivarius* PC-1 chromosomal DNA was partially digested with *Eco* R1 (Promega) and ligated to *Eco* R1-cut and dephosphorylated Lambda ZAP II vector arms (Stratagene) using T4 DNA ligase (IBI). The packaged recombinant bacteriophage particles were transfected into *Escherichia coli* XL1-Blue cells : *endAI, hsdR17* (rk-, mk+), *supE44, thi-1, lambda-, recA1, gyrA96, relA1, (lac-)* [F', *proAB, lacI^{q}ZΔ M15, Tn10* (*tet^R*)] (Stratagene). XL1-Blue was maintained on Luria-Bertani (LB)/tetracycline (12.5 μg ml^{-1}) (Sigma Chemical Co.) agar plates and routinely grown in LB medium (Maniatis *et al.*, 1982). Dextranase clones were identified as plaques surrounded by zones of clearing when plated in top agar containing blue dextran (Barrett *et al.*, 1987). A recombinant clone expressing dextranase activity, PD1, was selected for further study and stored in SM buffer (0.58% NaCl, 0.2% $MgSO_4$, 50 mM Tris, ph 7.5, and 0.1% gelatin) and chloroform. Clone PD1, was shown to carry a promoter and express a 190 KD polypeptide which exhibited dextranase activity by SDS-PAGE/electroblot, and blue dextran overlay (Lawman & Bleiweis, submitted, Journal of General Microbiology, 1991).

In vitro translation of PD1.

In vitro translation experiments were conducted using the prokaryotic DNA-directed translation kit from Amersham and L-[^{35}S] methionine (9.4 x 10^{-4} mM, 0.75 μCi μl^{-1}) (Amersham). This kit was used according to manufacturer's specifications and involved the addition of *E. coli* RNA polymerase, a mixture of unlabeled amino acids (without methionine), L-[^{35}S]-methionine, template DNA (either Lambda ZAP II DNA or PD1 DNA) and an *E. coli* cell extract. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 10%, 0.77 mm gels, at 25 mA using the discontinuous buffer system of Laemmli (1970). High range prestained SDS-PAGE standards (Bio-Rad Laboratories) were run simultaneously in order to determine the approximate molecular weight(s) of the recombinant protein(s). The gel was then fixed in 25% methanol (v/v) and 10% acetic acid (v/v), washed in dH_2O for 20 minutes, then in 1 M sodium salicylate for 20 minutes. After a brief rinse in dH_2O the gel was wrapped in plastic wrap and overlayed with X-ray film for 48 hours at room temperature before developing.

DNA manipulations, chemicals and enzymes.

Recombinant phage DNA of clone PD1 was prepared by a protocol for the large-scale preparation of bacteriophage lambda and subsequent extraction of DNA as described by Maniatis *et al.* (1982). DNA analysis was performed by horizontal agarose (SeaKem) gel (0.7% wt/v) electrophoresis in TAE buffer, pH 8.6 (40 mM Tris acetate, 1 mM EDTA) at 15V cm^{-1} of gel and visualized by the fluorescence of ethidium bromide. The restriction endonucleases; *Ssp* I, *Xmn* I, *Xba* I, *Not* I, *Aat* II, *Sac* I,

Sau I, *Sac* II, *Pvu* II, *Bal* I, *Bgl* I, and 10X universal restriction endonuclease buffer were obtained from Stratagene. T4 DNA ligase and the restriction endonucleases; *Tth* III 1, *Bam* HI, *Pst* I, and *Xho* I were obtained from IBI. *Pvu* I, *Hinc* II, *Nar* I, the nick translation kit, and PhotoGene™ nucleic acid detection systems were purchased from BRL, *Dra* III, *Sty* I, *Nhe* I, *Hind* III, *Nsi* I, *Eco* RI, and *Eco* RV were obtained from Promega. Biotin-labeled dATP was incorporated by nick translation into DNAs used as probes in the Southern hybridization analysis (either pPD13 or the 3.7 kb *Xho* I fragment of PD1) and were separated on PD-10 Sephadex G-25 M columns (Pharmacia). *Streptococcus salivarius* chromosomal DNA was extracted as previously described (Lawman & Bleiweis, submitted, Journal of General Microbiology, 1991), digested with restriction endonucleases, and separated by agarose gel electrophoresis, see above. After depurination in 0.25 M HCl (15 minutes), denaturation in 1.5 M NaCl and 0.5 M NaOH (twice for 20 minutes), and neutralization in 1.5 M NaCl and 1.0 M Tris-HCL, pH 7.5 (20 minutes), these DNAs were electroblotted onto PhotoGene™ nylon membranes (BRL) for 1 hour at 200 mA in 0.1X TAE buffer, pH 8.6. Biotinylated *Hind* III-cut lambda molecular weight markers (BRL) were run simultaneously. The Southern blots were prehybridized, hybridized with biotinylated probe DNA, and developed with PhotoGene™ nonradioactive detection system according to specifications from BRL. The blots were exposed to the PhotoGene™ substrate (15-60 minutes) and autoradiographed at room temperature (10 secconds-7 minutes) to obtain the desired results. All DNA manipulations using these products were conducted under the conditions suggested by the manufacturers.

Subcloning the *dex* gene.

In vivo excision and recircularization of the cloned inserts in the pBluescript plasmid were performed using R408 or VCSM13 as the f1 helper phages. Dextranase-positive plaques were cored from the blue dextran agar plate and transferred to a sterile microfuge tube containing 500 µl SM buffer and 20 µl chloroform. Each phage stock (200 µl) was incubated with 200 µl XL1-Blue cells and 1 µl helper phage (1×10^{11} pfu ml^{-1}). Five milliliters of 2X YT broth (1% NaCl, 1% yeast extract, and 1.6% bacto-tryptone) was added and incubated 3 hours at 37°C with shaking. The tube was heated at 70°C for 20 minutes. An aliquot of the rescued phagemid stock (10 µl) was incubated with 200 µl XL1-Blue cells at 37°C for 15 minutes. Infected XL1-Blue cells (50 µl) were plated on LB/ampicillin (50 µg ml^{-1}) (Sigma Chemical Co.) and incubated overnight at 37°C. Broth cultures of single colonies from the R408 excisions were tested for dextranase activity by placing samples in wells cut in a 1% agarose gel matrix containing 0.2% blue dextran and 50 mM potassium phosphate buffer, pH 6.35. The VCSM13 excisions produced no colonies.

The 6.3 kb insert of PD1 was excised with *Eco* RI, isolated by agarose gel electrophoresis, recovered by freezing and centrifugation in Microfilterfuge™ tubes with 0.45 µm nylon membrane filters (Rainin Instrument Co.), and ligated into *Eco* RI-cut pDL278 vector with T4 DNA ligase. The resultant recombinant plasmids were transformed into competent XL1-Blue cells following the procedure outlined by Maniatis *et al.* (1982). The transformation mixture was plated onto LB agar containing spectinomycin (50 µg ml^{-1}), 0.05 M isopropyl-β-thio-galactopyranoside (IPTG), and 5-bromo-4-chloro-3-indoyl-β-d-

galactopyranoside (X-gal), in N,N-dimethylformamide (DMF) (75 μg ml^{-1}). Plasmid DNA was extracted from the resulting white colonies by the boiling method supplied by Stratagene, digested with *Xba* I, and analyzed by agarose gel electrophoresis.

The 6.3 kb insert of PD1 containing the *dex* gene also was excised with *Xho* I and *Not* I, producing a 2.6 kb *Xho* I/*Not* I fragment and a 3.7 kb *Xho* I fragment. These fragments were subcloned into *Xho* I/*Not* I and *Xho* I-cut pBluescript vector (Stratagene), respectively. The ligation, transformation, and DNA extraction procedures used were as described above, except that ampicillin (50 μg ml^{-1}) was used for the selection of pBluescript. Large plasmid isolations of the succcessfully subcloned 2.6 kb *Xho* I/*Not* I fragment, pPD13, were prepared according to Maniatis *et al.* (1982) and purified by cesium chloride-ethidium bromide gradient-centrifugation in a VTi50 rotor at 45,000 rpm for 48 hours in a Beckman L7-55 Ultracentrifuge.

Results

Analysis of the *dex* gene product.

Previously (Lawman & Beiweis, submitted, Journal of General Microbiology, 1991), three polypeptides of molecular weights 190, 90, and 70 KD were shown to be responsible for the dextranase activity of PD1 by SDS-PAGE/electroblot and blue dextran overlays. Since it was not known whether the two lower molecular weight species were products of translation or proteolytic breakdown, an *in vitro* DNA-directed translation of PD1 was performed. The proteins encoded by PD1 were labeled with L-[^{35}S] methionine, analyzed by SDS-PAGE, and compared to similarly-

labeled Lambda ZAP II proteins. A major protein, 190 KD, was the immediate translation product synthesized by the recombinant (Figure 4-1). Neither the 90 nor the 70 KD polypeptide could be visualized in the *in vitro* translation of PD1. This experiment was performed in the absence of IPTG.

Subcloning the *dex* gene.

The Lambda ZAP II vector was designed to allow *in vivo* excision and recircularization to form a phagemid containing the pBluescript plasmid and the cloned insert. Broth cultures of single colonies were tested for dextranase activity (Figure 4-2b) and compared with activity in the original phage lysate before excision (Figure 4-2a). Dextranase activity was visualized by a zone of clearing surrounding the well. Unfortunately, the R408 helper phage could not be removed (Lawman & Bleiweis, unpublished results). When VCSM13 was used as the helper phage, excision was complete, but the insert containing the *dex* gene proved to be lethal to the *E. coli* host. Effort was made to subclone the entire 6.3 kb fragment into pDL278, an *E. coli/Streptococcus* shuttle vector (Dunny *et al.*, in press, Genetics and Molecular Biology of Streptococci, Enterococci and Lactococci, 1991) which was kindly provided by Dr. Don LeBlanc. However, use of this vector was unsuccessful. Attempts to subclone the 3.7 kb *Xho* I PD1 fragment also were unsuccessful, presumably resulting in lethality to *E. coli*. The 2.6 kb *Xho* I/*Not* I fragment; however, was subcloned into *Xho* I/*Not* I-cut pBluescript, where it was maintained stably. This construct, pPD13, was digested with numerous restriction endonucleases; the resultant map is shown in Figure 4-3. A deduced restriction map of PD1 is shown in Figure 4-4. Restriction endonucleases

which failed to cut within the 2.6 kb *Xho* I/*Not* I fragment were: *Eco* RI, *Bal* I, *Aat* II, *Sac* II, *Xmn* I, *Dra* III, *Bgl* I, *Xba* I, *Nar* I, *Nsi* I, *Tth* III 1, *Sau* I, and *Not* I.

Southern blot analysis.

Streptococcus salivarius PC-1 chromosomal DNA was completely digested with *Xho* I, *Xho* I and *Eco* RI, or *Xba* I (Figure 4-5a, lanes 2, 3, and 4, respectively). These digested DNAs were separated by agarose gel electrophoresis along with the appropriate controls; *Xho* I/*Not* I-cut pPD13 (Figure 4-5a, lane 5), *Xho* I/*Not* I-PD1 (Figure 4-5a, lane 6), and *Eco* RI-cut PD1 (Figure 4-5a, lane 7). These DNAs were blotted onto nylon membranes and probed with either biotin-labeled pPD13 (Figure 4-5b) or the 3.7 kb *Xho* I fragment from PD1 which had been gel-purified and nick translated to incorporate biotinylated dATP (Figure 4-5c). The pPD13 probe hybridized to a single 10.4 kb *Xho* I chromosomal fragment (Figure 4-5b, lane 2); a single 2.6 kb *Xho* I/*Eco* RI chromosomal fragment (Figure 4-5b, lane 3); a single 10.4 kb *Xba* I chromosomal fragment (Figure 4-5b, lane 4); both the 2.6 kb insert and the 3.0 kb pBluescript bands of pPD13 (Figure 4-5b, lane 5); the 2.6 kb fragment of *Xho* I/*Eco* RI-cut PD1 (Figure 4-5b, lane 6) from which it was subcloned; and a 6.3 kb fragment of the original PD1 clone (Figure 4-5b, lane 7). The 3.7 kb probe hybridized to a single 5.2 kb *Xho* I chromosomal fragment (Figure 4-5c, lane 2); a single 3.7 kb *Xho* I/*Eco* RI chromosomal fragment (Figure 4-5c, lane 3); a single 10.4 kb *Xba* I chromosomal fragment (Figure 4-5c, lane 4); the 3.7 kb *Xho* I/*Not* I fragment of PD1 (Figure 4-5c, lane 6); and the 6.3 kb *Eco* RI fragment of PD1 (Figure 4-5c, lane 7). This probe did not

hybridize to the pPD13 subclone to a significant degree (Figure 4-5c, lane 5).

Discussion

The extracellular endodextranase of *S. salivarius* has been shown to have an apparent molecular weight of 110 KD (Lawman & Bleiweis, submitted, Journal of General Microbiology, 1991). The corresponding recombinant form was expressed in *E. coli* as a 190 KD protein along with two smaller polypeptides (90 and 70 KD) which retained their ability to hydrolyze high molecular-weight blue dextran. It was not known if the 190 KD protein was the immediate product of translation, a conclusion that now can be made since a 190 KD protein results from the *in vitro* DNA-directed translation of PD1. Furthermore, it can be concluded that the 90 and 70 KD polypeptides were the result of *E. coli* proteolysis. The possibility remains; however, that the 190 KD protein may be a fusion product.

Attempts to subclone the 6.3 kb *Eco* RI fragment from PD1 and the 3.7 kb *Xho* I/*Eco* RI portion of the insert were unsuccessful, resulting in multiple rearrangements and/or lethality to the *E. coli* host. Others have been unable to isolate the RNA-polymerase sense strand of the *gtf B* gene (Shiroza *et al.*, 1987), the *ftf* gene (Shiroza & Kuramitsu, 1988), and the *scr B* gene (Sato & Kuramitsu, 1988) of *S. mutans* for sequencing. Each of these genes contained promoter sequences directly upstream from the structural gene and were expressed at high levels in *E. coli.* Clones containing the *dex* gene of *S. sobrinus* also are reported to be unstable (R. Curtiss III, personal communication). Perhaps the *dex* gene of *S. salivarius*

exhibits similar properties since it carries a promoter and can be expressed at high levels (see Figure 4-1). This also may account for the difficulty in obtaining *ftf* clones from the *S. salivarius*/Lambda ZAP II genomic library (Lawman & Bleiweis, unpublished results). The hope was that lethality might be controlled if the *dex* gene could be subcloned into the pDL278 shuttle vector which had been constructed to minimize the transcription of DNA inserts by surrounding the multiple cloning site with transcriptional terminators. This, however, did not seem to rectify the situation.

The 2.6 kb *Xho* I/*Not* I fragment was subcloned stably into pBluescript (see Figure 4-3). Since the *S. salivarius* insert in PD1 encodes a 190 KD dextranase, the expected composition of the *dex* gene should be approximately 5,100 bp, leaving at least 1,400 bp of the gene residing in the subclone, pPD13. It will be interesting to see if pPD13 contains the 3' or 5' sequence of the *dex* gene.

It has been postulated, based on mutational analyses, that *S. sobrinus* carries a single dextranase gene (Curtiss, 1985). To see if the same holds true for *S. salivarius* PC-1, chromosomal DNA was digested to completion with either *Xho* I, *Xho* I/*Eco* RI, or *Xba* I, and analyzed by Southern hybridization with both biotin-labeled pPD13 (carrying the 2.6 kb *Xho* I/*Not* I fragment of PD1) and the remaining 3.7 kb *Xho* I fragment (see Figure 4-5). It was known that the 6.3 kb *Eco* RI fragment of PD1 did not contain an *Xba* I restriction site (Lawman & Bleiweis, unpublished results); therefore, a single *Xba* I fragment of indeterminant size should carry the entire *dex* gene. This is the case, since both probes hybridized with a single fragment of 10.4 kb (see Figure 4-5b, lane 4 and 4-5c, lane 4). The pPD13 probe hybridized with a single 2.6 kb *Xho* I/*Eco* RI chromosomal fragment (see Figure 4-5b, lane 3), as predicted, and the 3.7 kb probe

hybridized with a single 3.7 kb *Xho* I/*Eco* RI chromosomal fragment (see Figure 4-5c, lane 3). Each probe hybridized to a single *Xho* I chromosomal fragment, each of unpredictable length (see lane 2 of Figure 4-5b and c). From these data it can be concluded that the only gene encoding the endodextranase of *S. salivarius* PC-1 has been cloned. It also is useful to know that genes linked immediately upstream and downstream would be readily available from an *Xho* I library, since a 10.4 kb *Xho* I clone could be selected by hybridization with pPD13 and would contain 7.8 kb of flanking DNA. Concomitantly, a 5.2 kb *Xho* I clone, selected by hybridization with the 3.7 kb fragment of PD1, would carry 1.5 kb of DNA flanking the other end of the *dex* gene.

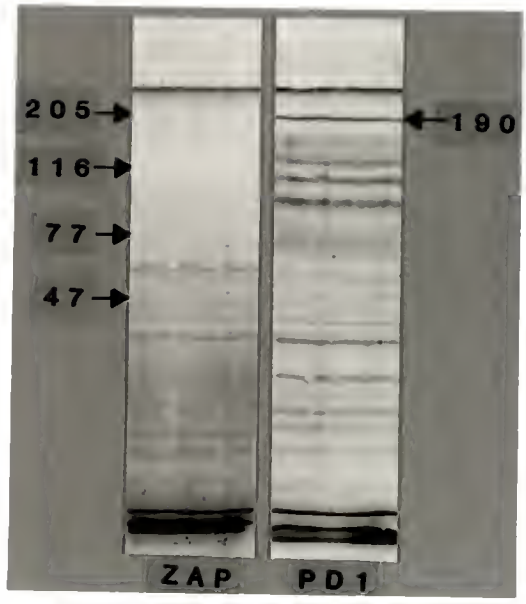


Figure 4-1. Autoradiographs of L-[^{35}S] methionine-labeled proteins from *in vitro* translations of Lambda ZAP II and PD1.

Positions of the molecular weight markers are shown on the left. A major protein band (190 KD) not expressed by the vector Lambda ZAP II was synthesized by the recombinant.

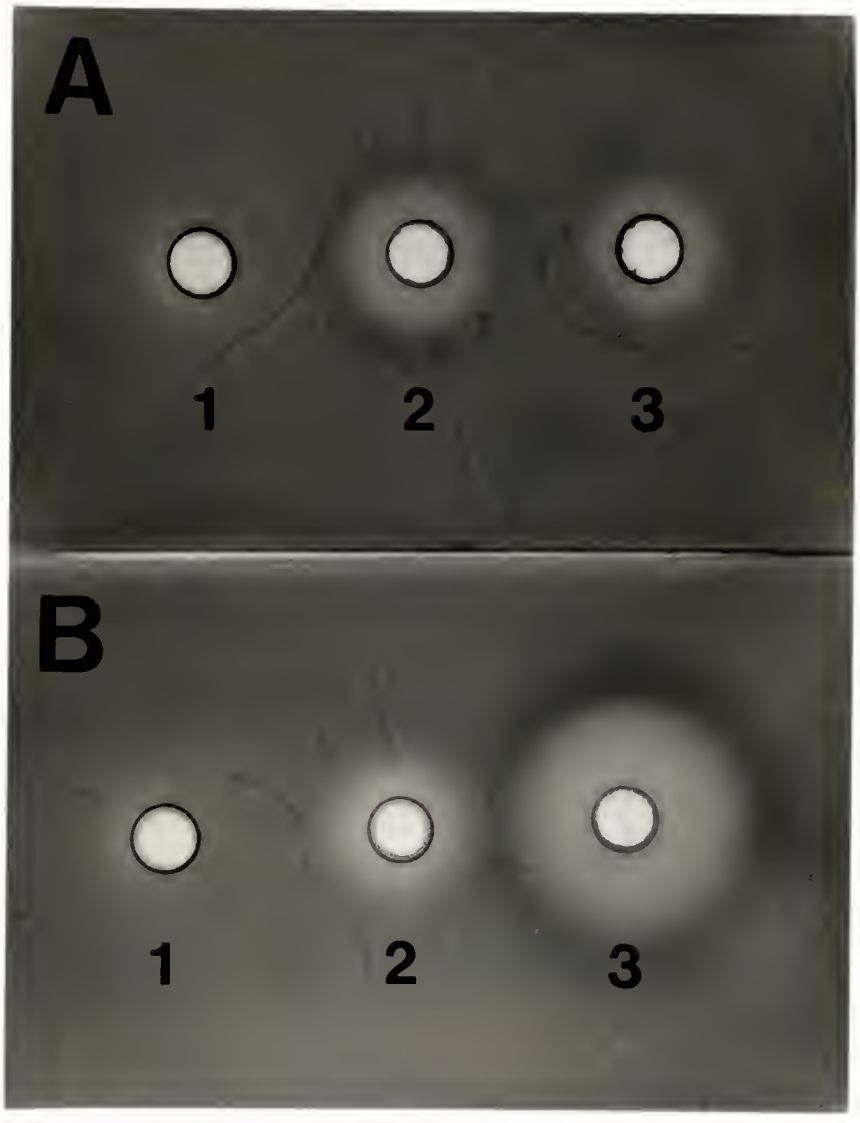


Figure 4-2. Excision of pBluescript and *dex* insert.

XL1-Blue cells were coinfected with PD1 and f1 helper phage R408 to produce phagmid constructs. XL1-Blue cells were infected with the packaged phagemid and plated on LB plates with ampicillin (50 $\mu g\ ml^{-1}$) to recover the excised plasmid. Broth cultures of single colonies were tested for dextranase activity (b) and compared with activity in the original PD1 phage lysates before excision (a). Samples were placed in wells cut in a 1% agarose gel matrix containing 0.2% blue dextran and 50 mM KPO_4 buffer, pH 6.35. Well 1 contains Lambda ZAP II/ no insert; wells 2 and 3, PD1.

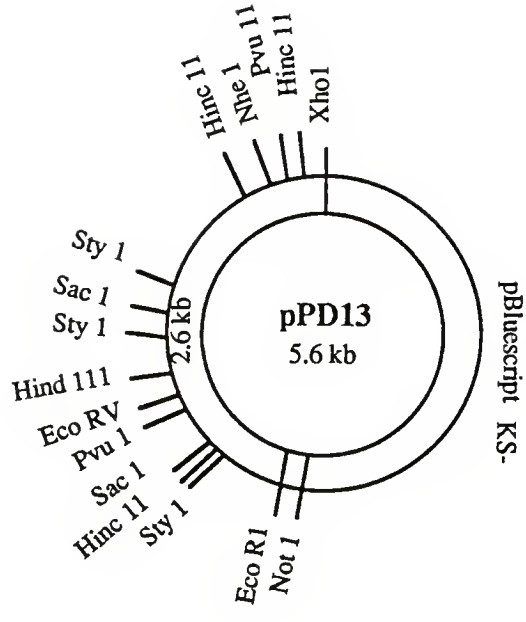


Figure 4-3. Partial restriction map of pPD13 containing the 2.6 kb *Xho* I/*Not* I fragment of PD1.

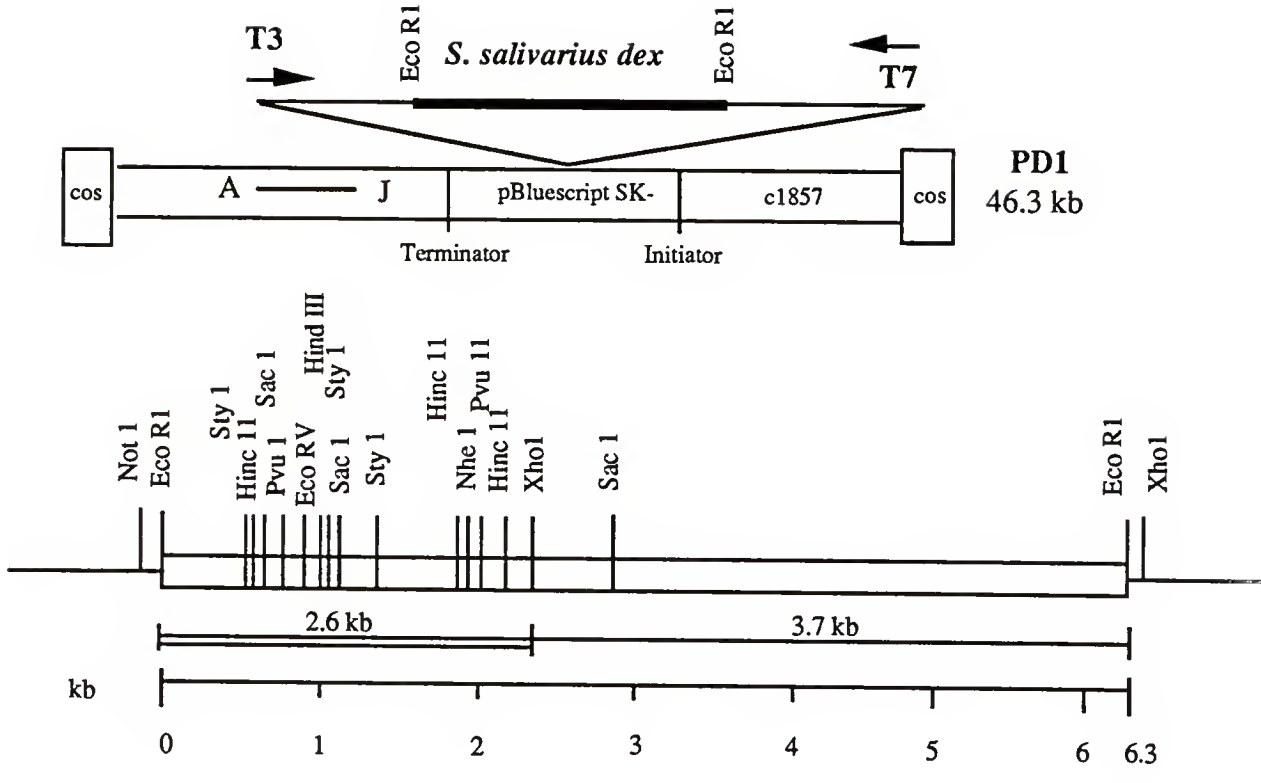


Figure 4-4. Construction of the original PD1 clone and deduced restriction map of the 6.3 kb fragment containing the *dex* gene.

The majority of restriction sites were mapped from the 2.6 kb *Xho* I/*Not* I fragment subcloned in pPD13.

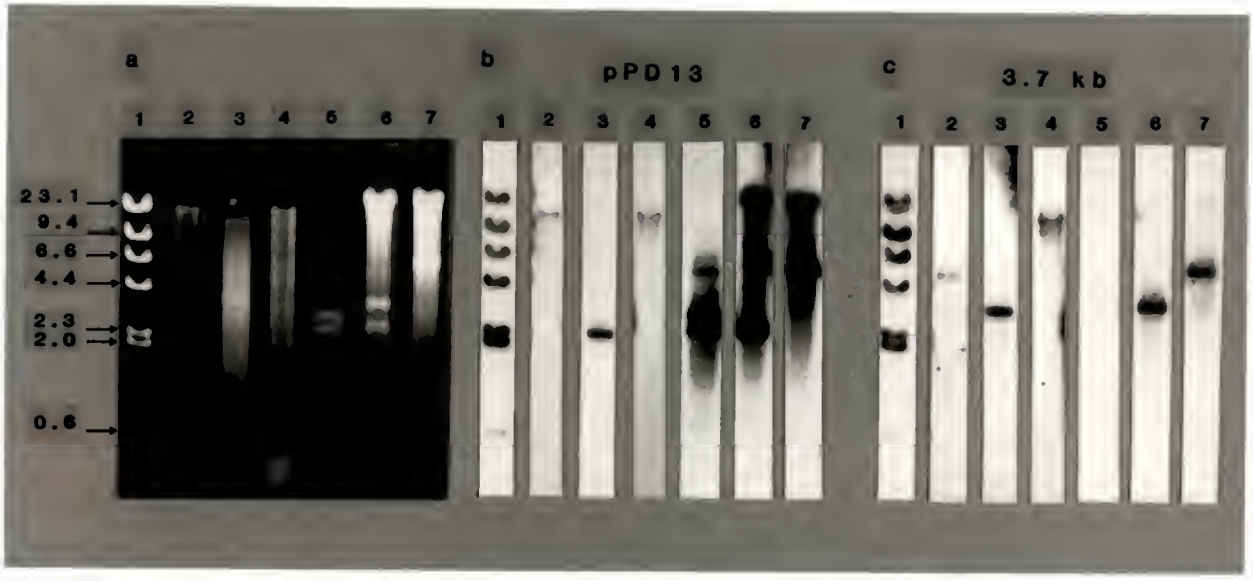


Figure 4-5. Southern blot analysis of complete restriction digests of the *S. salivarius* PC-1 chromosome.

Panel (a) represents the original agarose gel containing the following DNAs: lane 1, biotinylated *Hind* III-cut lambda molecular weight markers (the molecular weight of each marker is indicated to the left of panel (a); lane 2, *Xho* I-cut *S. salivarius* PC-1 chromosomal DNA; lane 3, *Xho* I/*Eco* RI-cut *S. salivarius* PC-1 chromosomal DNA; lane 4, *Xba* I-cut *S. salivarius* PC-1 chromosomal DNA; lane 5, *Xho* I/*Not* I-cut pPD13; lane 6, *Xho* I/*Not* I-cut PD1; and lane 7, *Eco* RI-cut PD1. Panels (b) and (c) represent the same DNAs blotted onto nylon membranes and probed with biotin-labeled pPD13 and the 3.7 kb *Xho* I fragment from PD1, respectively.

CHAPTER 5
SUMMARY

The dynamics of host-microbe interactions require adaptive responses on the part of the bacterium to diverse environmental conditions of different locations within the host, as well as the transition to and from an external reservoir (Miller *et al.*, 1989). This is especially true of *S. salivarius*, since this organism has developed the ability to colonize a wide range of mammalian species, can survive in the digestive tract and fecal matter, and can not only survive in the oral cavity, but outcompete many other species (Hardie, 1986). To survive encounters with such extremes in the immediate environment requires a constant sensing of the environment and a timely set of appropriate responses (Miller *et al.*, 1989).

Gram positive bacteria, including *S. salivarius*, must by necessity regulate major carbohydrate pathways differently from Gram negative organisms, since they have a much thicker cell wall, no outer membrane, and therefore, no periplasmic space (Volk *et al.*, 1986). Communication between the inside of the cell and the environmentally exposed exterior may be more difficult for Gram positive organisms. Therefore, it would be logical to hypothesize that some proteins destined for the outer surface of the cell wall might be produced at a baseline level, exported to the surface, and regulated at the posttranslational level. Such proteins might include large molecules, anchored to the wall, serving as scaffolds for extracellular enzyme networks. *Streptococcus salivarius* certainly has no

paucity of surface appendages (Gibbons *et al.*, 1972) and its extracellular proteins are difficult to separate from each other and from other structures such as lipids, teichoic acids, and peptidoglycan (Montville *et al.*, 1977). Both of these observations would support such a theory.

Sucrose can be considered an external stimulus to which inhabitants of the oral cavity must respond. *Streptococcus salivarius* has a number of extracellular and intracellular enzymes which are designed to metabolize sucrose, its constituents, and its byproducts (see Figure 1-1). To believe that these enzymes are not regulated in some manner seems to be untenable, and yet this was the accepted dogma until the present study began. Townsend-Lawman and Bleiweis (1991) were the first to show that sucrose-metabolizing enzymes in this oral *Streptococcus* are regulated, both genetically and posttranslationally. That these enzymes, e.g., GTF, FTF, fructanase, and dextranase are in fact regulated, was an easy hypothesis to make and yet quite a difficult one to test, since classical genetic techniques were and are largely unavailable for streptococci.

Since the overall objective of the preliminary physiological study was to determine whether or not one or more of these enzymes was differentially produced upon the addition of sucrose, appropriate culture conditions were developed first. Extensive growth curves were completed to determine the optimum stage of growth, where cells would be growing at a constant rate for a period of time, but not long enough to exhaust any nutrient. In this way growth rate would not be a variable factor in the production of these enzymes and repetitions of the experiments could be made. These curves were compared as the sole carbon source was varied (glucose, fructose, sucrose, galactose, or glucosamine). With this information, the growth conditions and cell preparations could be

normalized. The cells were filtered, washed, and reconstituted to a tenfold concentration equivalent with KPO_4 buffer. The supernatants were concentrated, dialyzed, and reconstituted to the same concentration with the same buffer. This gave cells at equivalent mass, growth phase, and concentration. Comparisons could then be made between cell-associated and extracellular enzymatic activities.

From the data presented in Chapter 1, it can be concluded that *de novo* synthesis is required for the production of extracellular GTF activity which, upon the addition of sucrose, becomes associated with the cell surface. Cell-associated FTF activity appears to require genetic induction for its production and cell-surface association, but requires sucrose for its release from the surface framework. Extracellular fructanase was twofold higher when cells were grown in sucrose than when they were grown in the other sugars. The increase in fructanase activity occurred within 5 minutes, but was diminished by transcriptional and translational inhibitors. Extracellular dextranase activity of cells grown in sucrose was tenfold higher than that of cells grown in glucose, fructose, or galactose. Dextranase activity increased 100-fold in less than 5 minutes following the addition of sucrose to galactose-grown cells. The increase in dextranase activity was affected by neither rifampicin nor chloramphenicol. Therefore, the production of dextranase activity by *S. salivarius* PC-1 does not require *de novo* synthesis.

Several lines of evidence suggest that dextranase is tightly controlled by a dextranase inhibitor which can be displaced by sucrose, and by one other factor, which appears to regulate and/or direct hydrolysis of dextran substrates. For example, sucrose was necessary for native dextranase activity and its effect was biochemically-mediated; supernatant from cells

grown in galactose inhibited recombinant dextranase activity, but when treated with sucrose the native enzyme acted synergetically with the recombinant enzyme; and recombinant dextranase released isomaltosaccharides from dextran substrates, while the native enzyme incompletely hydrolyzed the dextrans to large polymers.

Streptococcus salivarius PC-1 was able to utilize neither the substrates nor the products of dextran hydrolysis for growth. Therefore, the major biological role of the endodextranase appears to be the augmentation of GTF(s) in a synthetic capacity as opposed to being a catalytic convertor of stored dextrans.

Genetic analysis of the *dex* gene began as *dex* clones from a *S. salivarius*/Lambda ZAP II genomic library were identified by their ability to clear zones in blue dextran agar. A 6.3 kb *Eco* RI fragment from the genomic clone, PD1, was shown to carry a promoter and a sufficient portion of the *dex* structural gene to encode an active 190 KD recombinant protein. However, the native dextranase was represented by a 110 KD polypeptide. Russell and Ferretti (1990) found that the endodextranase activity of *S. mutans* could be localized in two major bands (120 and 105 KD) with breakdown products as low as 70 KD retaining their dextranase activity. Southern blot analysis using either end of the 6.3 kb *Eco* RI fragment showed that no other *S. salivarius* PC-1 chromosomal fragment had sufficient homology to the labeled probes to allow hybridization under the conditions used. From this it could be concluded that there is a single endodextranase gene in *S. salivarius*.

In order to sequence the *dex* gene it was necessary to amplify the cloned DNA. Attempts to subclone the 6.3 kb *Eco* RI insert were unsuccessful due to rearrangements and lethality to the *E. coli* host.

However, the 2.6 kb *Xho* I/*Not* I (*Eco* RI) fragment was subcloned stably into pBluescript (pPD13). This subclone was mapped by multiple digestions with restriction endonucleases.

There are certain predictions which can be made concerning the *dex* gene sequence prior to actually obtaining it, since sequence is the ultimate determinant of function and the sequences are known for several proteins with similar functions (see Tables 5-1 and 5-2), e.g., the *scr B* and *ftf* genes of *S. mutans* and the *sacA* and *sacB* genes of *Bacillus subtilis* all catalyze the transfer of fructose from sucrose and have homologous regions referred to as the "sucrose box" (Sato & Kuramitsu, 1988).

If 190 KD is the true molecular weight of dextranase, it is the largest of the sucrose metabolizing enzymes. It would be expected to have glucan-binding domains, a catalytic site, most likely a site for inhibitor binding, and perhaps a discrete site for the binding of a second regulatory factor which directs or limits the hydrolytic function. Due to the extracellular location of the protein and the ability to bind dextrans, dextranase may have extensive regions of homology with the GTFs and glucan binding protein (GBP) at the nucleotide and amino acid levels. It shares the large size and multifunctionality exclusively with the *gtf* genes. The *dex B, scr A, scr B,* and *gtf A* genes encode smaller proteins which are located within the cell, do not possess the long signal peptides typical of extracellular proteins and presumably have a single function. Fructosyltransferase is an extracellular enzyme which has a fructan binding domain, but must also contain a site for catalyzing the hydrolase reaction. It will be of interest to see if the active sites of the GTFs, FTF, fructanase, and dextranase are similar.

Since dextranase is an extracellular enzyme, it would be expected to have a signal sequence (Erm *et al.*, 1980). The signal sequences of most extracellular streptococcal proteins are longer than other bacterial signal peptides (Fahnestock *et al*, 1986; Hollingshead *et al.*, 1986). The average signal peptide of those extracellular enzymes listed in Table 5-2 (GTF-I's, GTF-SI, FTF, and GBP) consists of approximately 15 basic amino acids, followed by a stretch of hydrophobic amino acids, with the cleavage site at amino acid number 34, according to the rules set by von Heijne (1983). Putative ribosome binding sites have been defined by the sequences listed in Table 5-1 (Shine & Dalgarno, 1974). All the genes listed in Table 5-1, except for *gtf C* and *dex B*, which are thought to be internal to sugar transport and utilization operons, have been assigned promoter-like sequences according to comparisons with the *E. coli* promoter consensus sequence; $TTGACN_{17}$-TATAAT (Hawley & McClure, 1983).

The *dex B* gene of *S. mutans* does not carry its own promoter and is thought to be transcribed from the *gtf A* promoter which lies upstream of the *gtf A* structural gene, an ORF, and *dex B* (Russell & Ferretti, 1990). These authors found regions of similarity between this cytoplasmic exodextranase and other proteins attacking glucan polymers, i.e., pullulanase of *Klebsiella pneumoniae*; isoamylase of *Pseudomonas aeruginosa*; and neopullulanase of *B. stearothermophilus*.

The *gtf C* gene is preceeded by neither a termination-like sequence nor a promoter-like sequence and is thought to be coordinately expressed with the *gtf B* gene within a single operon from the *gtf B* promoter. These two genes share considerable homology with each other at the nucleotide- and amino acid-levels and by Southern hybridization. This homology spans three regions; an area near the 5' or N-terminus

corresponding to the signal sequence, a more central 2,000 nucleotide region with 95% homology, and a region at the 3' or C-terminal span of amino acids.

The 6.3 kb *Eco* RI fragment of PD1 appears to carry a promoter and the *dex* structural gene. However, if the 190 KD recombinant dextranase is a fusion protein there may be enough coding sequence within the insert to account for another gene with a promoter, even though such a protein was not seen by *in vitro* translation (see Figure 4-1). If not, the *dex* gene may either be transcribed alone or is located at the beginning of a coordinately regulated operon. It is difficult, however, to speculate as to what other genes might be located in such an operon, other than the dextranase inhibitor.

It has been noted that streptococcal genes often contain duplications, e.g., group A type M protein (Hollingshead *et al.*, 1986) and group B immunoglobulin G-binding protein (Fahnestock *et al.*, 1986; Guss *et al.*, 1986). This also is the case for some streptococcal genes whose proteins function in sucrose metabolism. The GTF-I of *S. mutans* has 3 repeats of 65 amino acids and a fourth of 36 amino acids, all located in the C-terminal end of the protein (Shiroza *et al.*, 1987). However, the C-terminus is not required for its activity, so the function of the repeats is unknown. GTF-SI contains 2 direct repeats of 49 amino acids, also at the C-terminus, function unknown (Ueda *et al.*, 1988). The GTF-I of *S. sobrinus* MFe28 has 6 repeating units of 35 amino acids of partial homology in the C-terminal one-third of the protein and 2 repeats of 48 amino acids of complete homology (Ferretti *et al.*, 1987). The function of these repeats has not been determined, however, GTF containing only the first two partial repeats retain the glucosyltransferase and glucan binding abilities. It has

been shown that the GBP has 6, A repeats (5 with 32-34 amino acids, and 1 with 12 amino acids) which show 48% identity (Banas *et al.*, 1990); and 4, C repeats of 17-20 amino acids with 78% identity. These repeats are staggered throughout the C-terminal three-quarters of the protein, show homology with the *gtf I, gtf B,*and *gtf C* genes, and are believed to be responsible for binding glucans. Helical regions may be able to form between the A repeats, but not within them (Banas *et al.*, 1990). It is not known if these structures are able to comply with the features described by Quiocho (1986) by forming a β-pleated sheet bounded by α-helical structures. Landale and McCabe (1987) were able to purify a polypeptide as small as 7.5 KD which still retained the ability to interact with glucan. It will be of interest to determine the correlation, if any, between the number of repeat units and the specificity of the binding reaction.

As the sequence data for dextranase becomes available, the degree to which it corresponds to the predictions based on other streptococcal genes related to sucrose metabolism can be ascertained. It should be possible to predict the molecular weight of the dextranase from the nucleotide sequence and determine if the 190 KD is a fusion protein. It may also be possible to determine this by Western analysis utilizing anti-β galactosidase antibodies. Future experiments also might include obtaining an N-terminal sequence of the mature and unprocessed dextranases to determine the exact cleavage site of the signal peptide. Such sequence information for the breakdown products able to retain dextranase activity would yield information about the location of the functional domains and the expendable regions of the protein. Now that the native and recombinant dextranases can be visualized by SDS-PAGE, it will be possible to purify sufficient quantities of these enzyme species to produce antibodies directed

against them. These antibodies could be used to study the kinetics of dextranase and dextranase inhibitor interactions, the possible binding of GTF or other regulatory molecules to dextranase, and the relationship of the *S. salivarius* dextranase with those from other species. With the availability of pPD13 as a nucleotide probe, it also will be possible to conduct Southern hybridization experiments to survey the distribution of homologous enzymes in other streptococcal species.

In conclusion, the work presented in this dissertation has laid the groundwork for the study of regulatory mechanisms in Gram positive organisms at many different levels. It has provided the experimental tools to explore the molecular biology of GTF(s), FTF, invertase, and other sucrolytic enzymes of *S. salivarius* by making available a comprehensive genomic library from which a number of these enzymes have already been cloned (see Chapter 3). Perhaps the most satisfying aspect has been the insight gained into the wonderfully complicated systems that the streptococci of the human oral cavity have developed to control such an extremely complex set of enzymes.

Table 5-1. Sequence analysis at the nucleotide level of streptococcal genes involved in sucrose metabolism.

Gene	Organism*	Shine-Dalgarno	-35	-10
gtf B	GS-5	GGAGG	TTGCAA-N17	TAGAAA
gtf C	GS-5	AGGA	-	-
ftf	GS-5	TAGGAT	ATGATA-N17	TAGGAT
scr A	GS-5	AGGAG	ATGATA-N17	AGTAAA
scr B	GS-5	AGGAG	TAGAAA-N17	TAGTAT
dex B	Ingbritt	AGGTGG	-	-
gtf A	Ingbritt	AGAGGA	CTGTTA-N16	TATAAA
gbp	Ingbritt	GGAGG	TTGAAA-N19	TATCTT
gtf I	MFe28	AGGAGGA	TTGACG-N18	TTAAAA

*GS-5 and Ingbritt are *S. mutans* strains and MFe28 is a *S. sobrinus* strain.

Table 5-2. Sequence analysis at the protein level of streptococcal genes involved in sucrose metabolism.

Protein	Suggested MW (D)	Suggested mature MW (D)	Apparent MW by SDS-PAGE (D)	Reference
GTF-I	165,800	162,300	155,000	Shiroza*et al.*, 1987
GTF-SI	153,000	149,000	155,000	Ueda *et al.*, 1988
FTF	87,600	83,000	91,000	Shiroza & Kuramitsu,1988
enzyme II^{Scr}	69,983	-	68,000	Sato *et al.*, 1989
Suc-6-PH	51,750	-	58,000	Sato & Kuramitsu,1988
exodextranase	62,103	-	62,000	Russell & Ferretti, 1990
Suc-6-Phos	55,665	-	55,000	Ferretti *et al.*, 1988
GBP	62,909	59,039	74,000	Banas *et al.*, 1990
GTF-I	177,100	172,983	173,000	Ferretti *et al.*, 1987

The proteins listed above correspond, in order, to the genes listed in Table 5-1.

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BIOGRAPHICAL SKETCH

Patricia Dianne Thibos was born September 23, 1952, in Chipley, Florida, where her parents, Ira and Minnie Lee Thibos continue to live. She attended Kate Smith Elementary School and Chipley High School, home of the Chipley Tigers. Upon graduation from high school in 1970, armed with a Florida State Board of Regents Scholarship and a Food Technology Scholarship, she went directly to the University of Florida. In 1972, she received an Associate of Arts and graduated with a Bachelor of Arts, with a major in anthropology, in 1973. For eleven years she devoted herself to a full-time career of rearing her three children; Josh, Jed, and Sommer. Making the decision to reenter the world of academia, she attended Jacksonville University, the University of North Florida, and the University of Florida in a postbaccalareate capacity to prepare for a graduate program in the sciences. In January, 1985, she entered the Department of Plant Pathology at the University of Florida, where the following year, she received a Master of Agriculture. She was accepted into the Department of Immunology and Medical Microbiology in January, 1987, this time with a Ford Foundation Doctoral Fellowship for Minorities. In the process of working for her Doctor of Philosophy, she met and married Michael John Patrick Lawman, to whom this dissertation is dedicated.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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